Effectiveness of 20 polymorphic microsatellite markers in parentage testing for water buffalo populations in Turkey

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ABSTRACT

In this study, 20 microsatellite markers were evaluated for parentage testing in Turkısh water buffalo populations. Despite the high polymorphism of the majority of 20 microsatellites an increase in homozygosity and deviations from Hardy Weinberg proportions were observed in some loci in the investigated populations. The twenty loci recommended by ISAG displayed high values for the measures of informativeness (allele numbers, heterozygosity, polymorphic information content, frequency of the most common allele, and power of discrimination). The number of alleles and expected heterozygosity (He) per marker ranged from 6 to 17, and from 0.444 to 0.881, respectively. The mean number of alleles (n_A) per locus was 9.50, the mean value of polymorphism information content (PIC) was 0.655, and the mean frequency of the most frequent allele (FNA) was 0.437. The most polymorphic microsatellite loci were: ETH003 (17 alleles, PIC= 0.788, FNA= 0.392), CSSM047 (14 alleles, PIC= 0.859, FNA= 0.224), CSSM045 (13 alleles, PIC= 0.869, FNA= 0.197). Combined power of discrimination (CPD) and combined power of exclusion (CPE) for the whole set of studied markers were 0.999. When both parents are known calculated combined probability of exclusion was at least 0.999.

INTRODUCTION

For sustained improvement in milk production, the dairy buffaloes are subjected to progeny testing under farm and field conditions. The successful and efficient use of a progeny testing program is a key factor for genetic improvement of dairy type buffalo breeds. Identification and exclusive use of proven sires is ideal to use them extensively for breeding programs; therefore, correct identification of sires in a breeding population is immensely important. However, wrong parentage information is a well-known problem in the estimation of breeding values of sires. Failure to record correct parentage can cause bias in the sire evaluation and introduce errors in estimates of heritabilities and breeding values. Therefore, verification of parentage may serve as a valuable tool for the success of progeny testing programs (Jakhesara *et al.*, 2012; Kathiravan *et al.*, 2012).

The aim of this study was to produce a paternity test kit for Anatolian Water Buffalo in the context of community based animal improvement program. It is intended to increase the efficiency of program by means of pedigree application and the success of animal breeding improvement projects with the resulting product of this project. With the help of paternity determination, animals with high genetic merit will be more precisely identified, these animals

will be selected from herds and bred to produce animals with higher productivity yield. In order to create elite herds of Anatolian Water Buffalo, which was included in animal breeding improvement programme in 2011, pedigree records created using accurate paternity testing can be achieved with the resulting kit of this project. At present, existed water buffalo herd management system no artificial insemination is practiced. Under present village conditions natural service usually only possible through the practice of hand mating, which is very labour intensive. According to the natural service practices; water buffaloes of the farmers of the same village are gathering and grazing on the their common public pasture to gather with several bulls as reproduction practices. This practice make difficulties of precise paternity identification from the pedigreed water buffalo improvement program. Alternatively, to allocate sufficient number of water buffalo for mating to one bull is not economically feasible due to increasing the cost of labour and other management cost. So, to apply paternity test by means of microsatellite based DNA test will be solution to overcome for this obstacle as decreasing the efficiency of improvement programs. Microsatellite based DNA testing, which is a worldwide accepted method, is routinely used for paternity testing for humans and applied for farm animals. With this method, the mother and the father of the animal is determined via statistical means with 99.73% accuracy, and the high productivity yielding animals are identified.

Microsatellite markers have been used extensively for parentage control in different species and are recommended by the International Society for Animal Genetics (ISAG) as they are highly abundant and informative, relatively inexpensive to use, and generate satisfactory results in tests for paternity exclusion (Luikart *et al.*, 1999; Arruga *et al.*, 2001; Curi and Lopes, 2002; Carneiro *et al.*, 2007; Glowatzki-Mullis *et al.*, 2007; Bolormaa *et al.*, 2008; Reis *et al.*, 2008; Carolino *et al.*, 2009; Araújo *et al.*, 2010; Stevanovic *et al.*, 2010; Zhang *et al.*, 2010; Adamov *et al.*, 2011; Saberivand *et al.*, 2011; Visser *et al.*, 2011). The present study was performed to evaluate the efficiency of a panel of 20 microsatellite markers in parentage testing of Anatolian water buffaloes.

MATERIALS AND METHODS

The study was based on a total 903 Anatolian buffalos (45 dams, 45 daughters and 15 sires, 798 unrelated individuals). Samples were collected from 6 different regions in Turkey (Marmara, Aegean, Black Sea, central Anatolia, Eastern Anatolia, Southeastern Anatolia). Genomic DNA was extracted from blood samples. Genomic DNA was extracted from 2 ml blood samples using High Pure PCR Template Preparation Kit (Roche, 2012). All DNA extraction and PCR amplification were performed by the Geometry Biotechnology (http://www.genometri.com.tr/) in Istanbul.

All studied water buffaloes were genotyped for 20 microsatellite markers located on different chromosomes (Table.1). The microsatellite markers analysis was performed using an Applied Biosystems 3130 Genetic Analyzer. The panel of microsatellite markers was designed recommended by ISAG/2010 – International Society by Animal Genetics/2010. The

microsatellite marker panels were grouped in four sets of fluorescent-labeled primers. Four primer pairs were used in each set for multiplex amplifications. The forward primer for each locus was labeled with one of the four fluorescent dyes FAM, HEX, and TAMRA (Applied Biosystems, USA). The PCR analyses were carried out using a T100^{TM} Thermal Cycler (Bio-Rad). The reaction mixture was composed of genomic DNA (100 ng), 200 mM dNTPs, 2.0 mM $MgCl_2$, 1X PCR buffer, 10 pmol forward and reverse primers and Taq DNA polymerase (0.5 u / sample). Polymerase chain reaction was performed with a total reaction volume of 25 µl using the following thermal conditions, 94 ^{0}C for 10 min, followed by 32 cycles of 94 ^{0}C for 1 min, 55^{0}C for 30 sec., 56 ^{0}C for 3 min and a final extension at 60 ^{0}C for 1 hour. Amplified DNA was verified by electrophoresis of PCR mixtures in 2% agarose gel in 1X TBE buffer. After electrophoresis for all microsatellites, allele size was determined on all samples with an ABI Prism® 3130 Genetic Analyzer (Applied Biosystems) using the GeneScan® Analysis Software (Applied Biosystems), which detects different alleles through size comparison with standard DNA size markers GeneScan 500 ROX Dye (Applied Biosystems).

Table 1. Information about the microsatellite markers used for parentage testing for water buffalo populations in Turkey.

Marker	Mix	Dye	Chr.	Allele range (bp)	GAN
CSSM061	1	HEX	Unkown(10)	99-125	**…**
CSSM033	1	FAM	17(17)[1]	151-171	U03805
ILSTS005	1	HEX	11(10)	173-189	L23481
CSSM022	1	FAM	4q(5)	245-267	U03806
CSRM060	2	FAM	11(10)	91-133	AF232758
BRN	2	HEX	11(10)	121-147	X59767
ILSTS033	2	TAMRA	13(12)	137-157	L37213
CSSM032	2	FAM	1q(1)	235-265	U03811
CSSM045	3	HEX	2q(2)	99-125	U03830
CSSM062	3	FAM	Unknown (6)	113-131	...
ILSTS030	3	HEX	2q(2)	153-169	L37212
BMC1013	3	FAM	3p(19)	292-314	G18560
CSSM057	4	FAM	9(7)	106-128	U03840
CSSME070	4	TAMRA	3p(19)	100-134	AF004364
CSSM036	4	FAM	1p(27)	149-179	U03827
CSSM043	4	HEX	1p(27)	173-215	U03824

ETH003	5	FAM	3p(19)	105-169	Z22744
CSSM047	5	TAMRA	3q(8)	115-169	U03821
CSSM029	5	FAM	9(7)	161-185	U03807
ETH121	5	HEX	2q(2)	181-209	Z14037

[1]Cattle chromosome assignments in parentheses. GAN; Genebank Accession Number

The number of allele (n_A), observed (H_O) and expected heterozygosity (unbiased – He) (Nei, 1978), polymorphic information content (PIC) (Botstein *et al.*, 1980), power of discrimination (PD) (Reis *et al.*, 2008; Cerit, 2003), probability of exclusion (PE) (Curi and Lopes, 2002; Cerit, 2003; Řehout *et al.*, 2006), and the paternity index (PI) were calculated for each microsatellite based on the parents' allele frequencies. These measures of informativeness were calculated using the Genetix (4.05) (Belkhir *et al.*, 1996 - 2000), GenAlEx 6 (Peakall and Smouse, 2006), Cervus 3.0 (Marshall, T., 1998/2006) and PowerStatsV12 programs (Brenner and Morris, 1990a). n_A, Ho and He were calculated as given by Nei (1978, 1987), and PIC as was formulated by Botstein *et al.* (1980). PD was calculated as was by Kimberly (2001) and as defined by Brenner and Morris (1990b). Combined power of discrimination (CPD) for n loci was also calculated (Kimberly, 2001; Brenner and Morris, 1990b). PE was defined for three alternative cases (Jamieson, 1994; Jamieson and Taylor, 1997); PE1 estimates the probability of exclusion of a parent when genotypes of the offspring and both its parents are known; PE2 estimates the probability of exclusion of a parent when genotypes of the offspring and only one of its parents is known; and PE3 estimates the probability of excluding two putative parents when genotypes of the offspring and both of its parents are known (Jamieson, 1994; Jamieson and Taylor, 1997). Combined probabilities of exclusion (CPE) over n unlinked loci in all three of the above cases were also calculated (Jamieson and Taylor, 1997). To calculate paternity index, the genotype frequencies of the putative parents were examined for each locus. For the 15 candidate's bulls, only the genotypes of fathers were available. Appropriate formulae given by Morris (1983) were used to calculate the paternity indices. Combined paternity index was calculated in accordance with Morris' (1983) article, as it was referred by Brenner and Morris (1989) and Ostrowski (2006). Finally, probability of paternity (POP) was calculated as given by Morris (1983).

RESULTS AND DISCUSSION

In this study, 20 microsatellite loci were first evaluated with respect to their informativeness in terms of the number of allele (n_A), observed heterozygosity (H_O), expected unbiased heterozygosity (H_e), polymorphic information content (PIC), frequency of the most frequent allele (FNA), power of discrimination (PD) and probability of exclusion (PE) in Anatolian water buffalo population. Loci and their different measures of informativeness are presented in Table 2.

The informative loci are used to confirm the parentage testing within a group of 903 Anatolian water buffalo individuals in Turkey. Effectiveness of parentage testing depends primarily on the level of informativeness (or effectiveness; power for identifying the individuals uniquely) of the used loci.

In the present study, the number of alleles per locus ranged from 6 (ILSTS005) to 17 (ETH003). The mean number of alleles per locus was 9.50 and the total number of alleles was 190 (Table 2). The highest number of alleles was observed for ETH003 (17), CSSM047 (14) and CSSM045 (13). All also have high values in other measures (high He, high PIC and high PD).

Informativeness level of a locus depends on the number of alleles exhibited by the locus and the frequency distribution of these alleles in the population (the more even the distribution of the alleles, the higher the informativeness of the locus). Among all of the loci examined, frequency of the most frequent allele was lowest in CSSM045 (0.197), making CSSM045 the most informative (e.g. PIC=0.869) locus.

The observed heterozygosity and the expected heterozygosity ranged 0.319 - 0.779 (the average value was 0.608) and 0.444-0.881 (the average value was 0.693) in the Anatolian water buffalo, respectively.

Table 2. Microsatellites used, observed sizes of their alleles, number of alleles (n_A), observed heterozygosity (H_O), expected heterozygosity (H_e), frequency of the most frequent allele (FNA), polymorphism information content (PIC), Probability of Exclusion (PE) and power of discrimination (PD) values in Anatolian water buffalo population in Turkey.

Locus	n_A	H_0	H_E	H_0-H_e	FNA	PIC	PD	PE_1	PE_2	PE_3
CSSM061	9	0.766	0.810	-0.044	0.338	0.787	0.935	0.638	0.462	0.823
CSSM033	7	0.367	0.444	-0.077	0.726	0.413	0.630	0.251	0.104	0.410
ILSTS005	6	0.598	0.568	0.030	0.478	0.473	0.654	0.275	0.164	0.411
CSSM022	8	0.587	0.670	-0.083	0.500	0.627	0.834	0.441	0.267	0.634
CSRM060	10	0.701	0.729	-0.028	0.455	0.699	0.893	0.530	0.346	0.732
BRN	9	0.497	0.595	-0.098	0.595	0.557	0.767	0.377	0.205	0.570
ILSTS033	7	0.579	0.645	-0.066	0.547	0.608	0.829	0.424	0.244	0.621
CSSM032	8	0.319	0.445	-0.126	0.735	0.428	0.612	0.274	0.112	0.455
CSSM045	13	0.648	0.881	-0.233	0.197	0.869	0.963	0.763	0.615	0.915
CSSM062	10	0.657	0.823	-0.166	0.272	0.800	0.938	0.650	0.480	0.830
ILSTS030	7	0.543	0.612	-0.069	0.474	0.537	0.766	0.338	0.200	0.500
BMC1013	9	0.624	0.780	-0.156	0.331	0.749	0.916	0.582	0.404	0.770
CSSM057	8	0.721	0.775	-0.054	0.261	0.738	0.911	0.556	0.380	0.740

CSSME070	10	0.681	0.837	-0.156	0.212	0.816	0.928	0.672	0.501	0.844
CSSM036	10	0.688	0.654	0.034	0.432	0.592	0.789	0.400	0.243	0.580
CSSM043	8	0.536	0.618	-0.082	0.566	0.576	0.803	0.390	0.220	0.578
ETH003	17	0.617	0.804	-0.187	0.392	0.789	0.925	0.654	0.480	0.851
CSSM047	14	0.779	0.872	-0.093	0.224	0.859	0.962	0.750	0.593	0.904
CSSM029	10	0.554	0.535	0.019	0.600	0.458	0.689	0.272	0.150	0.415
ETH121	10	0.711	0.760	-0.049	0.401	0.731	0.905	0.563	0.382	0.760
Combine							0.9999	0.9999	0.9997	0.9999
Mean	9.5	0.608	0.693		0.437	0.655				

PE_1=Probability of Exclusion (Both parents known), PE_2=Probability of Exclusion (Only one parent is known); PE_3=Probability of Exclusion (Both parents known, exclude two putative parents) (Jamieson and Taylor, 1997).

In summary, among the tested 20 loci CSSM045, CSSME070, CSSM047, CSSM057 and CSSM062 emerged as the most useful loci in parentage analysis of Anatolian water buffalo in Turkey. Difference between observed and expected heterozygosities revealed deviation from Hardy-Weinberg equilibrium. The range of deviation – 0.233 (CSSM045) / - 0.028 (CSRM060) was smaller than the range of reference studies (e.g. Maichomo *et al.*, 2008; Řehout *et al.*, 2006). Differences may be due to the presence of null alleles or genotyping errors. As 17 out of 20 Ho – He differences have negative values, it can be said that high degree of observed homozygosity is not due to the presence of null alleles (Allendorf and Luikart, 2007). There was no single locus discrepancy between the parents and their offspring, suggesting very low levels of genotyping error. Hence differences can be attributed primarily to the deviation of the randomly selected genotypes from those of Hardy–Weinberg expectations.

Between the Indian and Anatolian water buffalo populations there were minor (±1–3) differences in the number of observed alleles for these loci. E.g., in Anatolian water buffalo CSSM61 exhibited 9 alleles but 12 alleles were observed in the Indıan Mehsana buffalo population (Jakhesara, *et al.*, 2012; Kathiravan *et al.*, 2012). Calculated PIC values ranged from 0.413 for marker CSSM033 to 0.869 for marker CSSM045. Of the 16 microsatellite markers, except CSSM033, ILSTS005, CSSM032 and CSSM029, were highly informative with PIC values of more than 0.5.

In the present study, power of discrimination ranged from 0.612 (CSSM032) to 0.963 (CSSM045). Combined power of discrimination value (CPD) for the 20 microsatellite loci was 0.9999, which is the required level of discrimination in a parentage analysis (Vankan and Faddy, 1999; Pérez-Miranda *et al.*, 2005). After verifying the informativeness of 20 tested loci probability of exclusion for each loci was calculated. The probability of exclusions, PE_1, PE_2 and PE_3 as defined in the Materials and methods section are shown in Table 2. Table 2 indicates that CSSM045 and CSSM047 are the two loci exhibiting the highest PE values (PE_1, PE_2, PE_3) in the

studied population. Other loci with high PE values are: ETH003, CSSME070, CSSM062 and CSSM061.

When genotypes of the individual and one of its parents are known, the combined paternity index is low (CPE_2=0.9997), lower than the required value. When both of its putative parents were known probability of exclusion for one of the parents, CPE_1, is0.999. Under the same scenario probability of excluding both parents (e.g. the probability of detecting the substituted individual) reaches a higher value: CPE_3=0.9999. Calculated PE and CEP values are again comparable to those given by Kathiravan *et al.,* 2012 and higher than those given by Jakhesara *et al.,* 2012.

In the present study was to develop and test a suitable multiplex panel consisting of microsatellites for parentage verification in Anatolian water buffalo. These results suggest that multiplex microsatellite panel is a fast, robust, reliable, and economic tool to verify the parentage as well as to assign the putative sire to daughters under progeny testing with very high accuracy and hence can be used in routine parentage testing. The combined PE value of all 20 microsatellites was 0.999, ensuring parentage assignments with a 99.73 % confidence level. These microsatellites are highly polymorphic and have proved very useful for parentage testing in the Anatolian water buffalo population.

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