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Exclusion Probabilities of 8 DNA Microsatellites in 6 Cattle Breeds from Northeast Mexico

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Abstract: Traditionally, parentage verification in cattle has been performed using blood typing. Parentage verification is very important, since it is the basis for breeding schemes and becomes even more critical with the widespread use of artificial insemination and embryo transfer techniques. Recently, a new methodology employing DNA markers is being used for parentage control. A panel of 9 DNA microsatellite markers was chosen internationally for evaluation in the cattle population of different countries. In order to achieve this goal, information regarding the amount of genetic variation of the microsatellites must be obtained. In the present study, the number of alleles and their frequencies were determined in 8 microsatellites from the panel (BM 1824, BM 2113, SPS 115, ETH 3, ETH 10, ETH 225, TGLA 122 and TGLA 227) using PCR and genotyping. This data was then used to calculate heterocigozity, polymorphism information content and exclusion probabilities of the microsatellites, both individually and as a group. A total of 287 animals belonging to 6 cattle breeds (Holstein, Simmental, Brown Swiss, Beefmaster, Brahman and Brangus) and localized in northeast Mexico were studied. In the Simmental breed, 3 of the 8 microsatellites were not analyzed (BM 1824, ETH 3 and TGLA 122). Considering the 8 microsatellites, exclusion probabilities were as follows: Holstein, 0.9988; Simmental, 0.9924; Brown Swiss, 0.9998; Beefmaster, 0.9999; Brahman, 0.9930; Brangus, 0.9990. All of the eight genetic markers in the 6 cattle breeds analyzed, except BM 1824 and ETH 3 in Brahman (exclusion probabilities of 0.3800 and 0.2200, respectively), as well as SPS 115 in Holstein (exclusion probability of 0.1213) were highly informative and their exclusion probabilities as a group demonstrate their usefulness for parentage verification in cattle from northeast Mexico.

Key words: Bovine, parentage verification, DNA microsatellites

INTRODUCTION

Since, 1980, parentage testing in cattle has been done by blood typing, but it was replaced in the mid-90's for the so called DNA test or genotyping. The simultaneous use of several genetic markers, named DNA microsatellites, allows the achievement of a higher than 99.9% reliability for genealogy verification. It has been observed than with the use of 22 microsatellites, an exclusion probability of 0.9999 can be obtained, which means that the probability of excluding as progenitor an individual that is not the actual progenitor is of 99,999%. Laboratory materials and protocols for genotyping are easily available, results are comparable among different laboratories and different biological samples can be used for analysis (Glowatzki-Mullis *et al*., 1995; Heyen *et al*., 1997; Vankan and Faddy, 1999).

To determine the degree of usefulness of microsatellites for parentage testing in a certain population, the number and frequency of their alleles must be evaluated. With this information it is possible to calculate their heterozygosis and degree of polymorphism, which in turn allows the determination of the exclusion probability of each individual microsatellite, as well as of a panel of microsatellites as a group. Exclusion probability indicates the possibility of excluding as progenitor an individual assigned as such incorrectly. Normally it is proven that it is impossible to exclude a particular individual as sire or dam of a calf and the animal is said that qualifies as progenitor and can be certified as such in the pedigree papers. In other occasions, an assumed progenitor can be excluded as such with a probability of up to 0.9999 (Caldwell, 1996).

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Recent studies in cattle have used microsatellite analysis to determine genetic diversity among breeds (Hansen *et al*., 2002; Maudet *et al*., 2002), to assign paternity in legal disputes (Liron *et al*., 2004) and to determine the degree of mistakes existing in genealogy records and its impact in animal breeding programs; the error rate found was of about 10% (Banos *et al*., 2001; Israel and Seller, 2000; Visscher *et al*., 2002). This confirms the importance of paternity tests for accurate pedigree data. Also, new statistical methods have been developed to calculate exclusion probabilities based in microsatellite analysis in cases with more than one candidate sire, as happens with multiple sire programs (Neff *et al*., 2000, 2001; Sherman *et al*., 2004).

In the present study, it was determined the number of alleles and their frequencies of 8 microsatellites (BM 1824, BM 2113, SPS 115, ETH 3, ETH 10, ETH 225, TGLA 122 and TGLA 227). Also, their heterozygosis, Polymorphism Information Content (PIC) and Exclusion Probabilities (EP), both individually and as a group were calculated. Six different cattle breeds were included (Holstein, Simmental, Brown Swiss, Beefmaster, Brahman and Brangus). The microsatellites analyzed belong to the panel chosen by the International Society of Animal Genetics (ISAG) (Caldwell, 1996).

MATERIALS AND METHODS

Blood samples were obtained and DNA was extracted by the salting-out method from 287 bovines: 39 Holstein, 38 Simmental, 54 Brown Swiss, 59 Beefmaster, 47 Brahman and 50 Brangus, located in ranches at northeast Mexico (states of Nuevo Leon, Coahuila and Tamaulipas). The fluorescent marker, concentration of reagents, PCR primer sequence and grouping of reactions for multiplex PCR are shown in Table 1. Eight microsatellites were analyzed, except in the Simmental breed, in which only 5 were included due to lack of good PCR amplification. These microsatellites have been analyzed in other cattle population and were selected for their high heterozygosis, chromosomal location, wide range of sizes and easiness to be amplified in multiplex PCR reactions (Caldwell, 1996). The forward primer of each microsatellite was labeled with fluorescence according to Table 1.

Multiplex PCR reactions were performed in a reaction mix of 25 μL containing 40 ng genomic DNA, 1 x PCR buffer (including $MgCl_2$), 200 μM each dNTP and 1.25 units of Taq polymerase. Primer concentrations are shown in Table 1. Guidelines for PCR amplification improvement of Henegariu *et al*. (1997).

Termocycler (M. J. Research 100) program was as follows: initial denaturalization at 94°C, 4 min; 30 cycles of denaturalization at 94°C, 4 min, hybridization at 56°C, 1 min and extension at 72°C, 3 min; a final extension at 72°C, 7 min, with the exception of multiplex 2, in which hybridization temperatures were 60°C for ETH 10 and ETH 225 and of 66°C for ETH 3. PCR amplification was confirmed by 2% agarose gel electrophoresis at 75 V by 1 h, including a molecular size marker and negative control. The length (in base pairs or b.p.) of PCR products were determined with a DNA sequencer (ABI Prism 373A, Applied Biosystems) using the Genotyper 1.1 program, which automatically determines the fragment sizes according with the internal size marker (TAMRA).

The allele number of each microsatellite was determined by direct count. Genic frequency for each microsatellite was calculated by dividing the number of times each allele was present among the number of times all alleles were present.

Heterozygosis was calculated by the following formulae:

$$H = 1 - \Sigma P1^2$$

were, Pi is the frequency of allele I (Heyen *et al*., 1997; Usha *et al*., 1995).

Table 1: PCR multiplex conditions

Múlti-plex	Locus	Label	Concentration	PCR primer
1	BM 1824	ROX	0.25 μM	I1: GAG CAA GGT GTT TTT CCA ATC
				I2: CAT TCT CCA ACT GCT TCC TTG
	BM 2113	TET	0.25 μM	I1: GCT GCC TTC TAC CAA ATA CCC
				I2: CTT CCT GAG AGA AGC AAC ACC
	SPS 115	HEX	0.25 μM	I1: AAA GTG ACA CAA CAG CTT CTC AG
				I2: AAC GAG TGT CCT AGT TTG GCT GTG
2	ETH 10	TET	0.3 μM	I1: GTT CAG GAC TGG CCC TGC TAA A
				I2: CCT CCA GCC CAC TTT CTC TTC TC
	ETH 225	HEX	0.3 μM	I1: GAT CAC CTT GCC ACT ATT TCC T
				I2: ACA TGA CAG CCA GCT GCT ACT
3	TGLA 122	ROX	0.4 μM	I1: CCC TCC TCC AGG TAA ATC AGC
				I2: AAT CAC ATG GCA AAT AAG TAC ATA C
	TGLA 227	HEX	0.4 μM	I1: CGA ATT CCA AAT CTG TTA ATT TGC T
				I2: ACA GAC AGA AAC TCA ATG AAA GCA
4	ETH 3	ROX	0.6 μM	I1: GAA CCT GCC TCT CCT GCA TTG G
				I2: ACT CTG CCT GTG GCC AAG TAG G

PIC value was calculated with the next formulae:

$$1-(\sum_{i=1}^{n} Pi^2)\ \sum_{i=1}^{n-1}\ \sum_{j=1+1}^{n} Pi^2\, pj^2$$

were, Pi is the frequency of i-esim allele (Usha *et al*., 1995).

Exclusion probability, which indicates probability that an alleged parent be excluded as potential progenitor, was calculated with following formulae, which assumes it is an individual taken at random:

$$EP = 1-\prod_{i=1}^{N} qi$$

where:

qi = The exclusion probability of marker.
I and N = The total number of markers (Heyen *et al*., 1997; Usha *et al*., 1995).

RESULTS AND DISCUSSION

Three multiplexes and one single PCR reactions were evaluated for parentage verification purposes. Table 2 shows the observed number for each allele. From this data were calculated allele number and frequency, size, heterozygosis, PIC and exclusion probabilities. Allele number had a considerable variation, from 4 for ETH 3 in Brahman to 22 for BM 2113 in Beefmaster. Markers with a low allele number were ETH 3 in Brahman (4), ETH 10 in Simmental and Brahman (6 and 5, respectively), ETH 225 in Simmental (5) and TGLA 227 in Brahman (6). The markers with the highest allele number were BM 2113 in Beefmaster and Brangus (22 and 18), ETH 3 in Holstein (19), as well as ETH 10 (18) and ETH 225 (21) in Brangus.

With regard to heterozygosis, markers with the lowest values were SPS 115 in Holstein, ETH 3 in Brahman and ETH 225 in Beefmaster (0.4132, 0.5179 and 0.5887, respectively), whereas the highest values were BM 2113 in Simmental, Beffmaster and Brangus, ETH 225 in Brangus and TGLA 227 in Simmental (0.8739, 0.8811, 0,8855, 0.8799 and 0.9301, respectively) (Table 3).

PIC values were very high in all markers and breed; of the 46 PIC values obtained, 12 had the maximum value of 1. Six of the obtained exclusion probabilities had values <0.5: BM 1824 in Brahman (0.38), SPS 115 (0.1213) and ETH 3 (0.4524) in Holstein, ETH 3 in Brahman (0.22), ETH 19 in Brangus (0.45), as well as ETH 225 in Simmental (0.4155). These values, when considered independently, could indicate that they may not be useful for paternity testing; however, considering all markers for each breed in order to obtain the combined exclusion probability, we found that only in Simmental and Brahman was low (0.9924 and 0.9930). In the Simmental breed this is due to the fact that only five markers were analyzed, whereas in Brahman is because to the low exclusion probability of some markers (BM 1824 and ETH 3). The other markers in this breed had not so high exclusion probability values. Therefore, it could be necessary to substitute BM 1824 and ETH 3 for other markers in the Brahman breed. Total PE for each breed was: Holstein: 0.9988; Simmental: 0.9924; Brown Swiss: 0.9998; Beefmaster: 0.9999; Brahman: 0.9930; Brangus: 0.9990 (Table 3).

The present study shows, evidence that parentage testing using DNA markers in cattle from northeast Mexico gives an acceptable degree of reliability, such as has been demonstrated in other cattle populations. The DNA markers chosen by the ISAG are therefore convenient for use with the local cattle population. Results obtained demonstrate that the described methodology constitutes a useful, fast and trustworthy way to perform parentage tests in Mexico. The 8 DNA microsatellites used allow the exclusion in the analyzed breeds, on average, of 99.8% of individuals that are not progenitors of a particular animal. This can be applied when only the genotype of the offspring and of the alleged parent is known (Usha *et al*., 1995).

For the Brahman breed, which had the lowest exclusion probability with 8 markers, it may be convenient to analyze a set of microsatellites different from the ones used in this study. This will also, be necessary for the Simmental breed, in which only 5 markers were analyzed.

Some not previously reported alleles were found, since the size rank of markers ETH 3, ETH 10, ETH 225, TGLA 122 and TGLA 227 in this study is different to the ones reported. For example, TGLA 122 has been reported with a size rank of 135-181 bp, whereas we found its size rank varied from 128-175 bp. Also, the smaller size informed for TGLA 227 was 78 bp, but in this study we report an allele of 66 bp (Heyen *et al*., 1997).

DNA microsatellite polymorphisms are formed by mutations, which originate new alleles, differing from each other in the number of reported sequences. Therefore, care must be taken when a progenitor is to be disqualified as a parent based only in one marker, since the difference in the number of repetitions could be due to a mutation and not to the fact that it is not the real progenitor (Glowatzki-Mullis *et al*., 1995; Heyen *et al*., 1997).

Data in allele number, heterozygosis, PIC and EP values found in this research are in general similar to those reported by Glowatzki-Mullis *et al*. (1995), Heyen *et al*. (1997) and Usha *et al*. (1995).

Previous studies have shown that pedigree errors, that can be as high as 10% (Visscher *et al*., 2002),

Table 2: Characterization of the 8 microsatellites in the 6 bovine breeds

Locus	Breed	N	No. of alleles	Size rank	Hetero-cygozity	PIC	P.E.
BM 1824	Holstein	63	9	175-190	0.7732	0.9991	0.5861
	Brown swiss	108	9	176-188	0.6921	1	0.7075
	Beefmaster	100	9	178-189	0.7470	1	0.5570
	Brahman	108	9	177-192	0.6294	0.9998	0.38
	Brangus	94	10	150-188	0.7264	0.9983	0.52
BM 2113	Holstein	68	10	126-144	0.8345	0.9992	0.6209
	Simmental	74	14	129-145	0.8739	0.9962	0.7500
	Brown swiss	108	15	128-146	0.8245	1	0.7966
	Beefmaster	102	22	127-150	0.8811	1	0.9187
	Brahman	54	12	129-148	0.8576	0.9999	0.72
	Brangus	88	18	108-149	0.8855	0.9996	0.77
SPS 115	Holstein	56	9	245-262	0.4132	0.9443	0.1213
	Simmental	60	11	250-265	0.7983	0.9934	0.6247
	Brown swiss	108	11	223-260	0.7191	1	0.6424
	Beefmaster	46	14	240-262	0.8582	1	0.7266
	Brahman	60	11	140-156	0.7155	0.9996	0.54
	Brangus	90	17	227-262	0.8540	0.9999	0.72
ETH 3	Holstein	70	19	92-126	0.6621	0.9996	0.4524
	Brown swiss	78	9	90-124	0.7646	0.9980	0.5752
	Beefmaster	50	11	99-124	0.8238	0.9990	0.6819
	Brahman	98	4	100-114	0.5179	0.9493	0.22
	Brangus	84	17	91-124	0.8398	0.9998	0.69
ETH 10	Holstein	72	14	105-229	0.7862	0.9994	0.6045
	Simmental	44	6	214-226	0.7827	0.9908	0.6287
	Brown swiss	74	13	213-230	0.8397	1	0.6835
	Beefmaster	50	14	206-226	0.7892	1	0.6717
	Brahman	54	5	213-225	0.6879	0.9686	0.50
	Brangus	88	18	173-225	0.8380	0.9990	0.45
ETH 225	Holstein	70	10	114-154	0.7134	0.9925	0.69
	Simmental	51	5	142-162	0.7058	0.9809	0.4155
	Brown swiss	88	8	142-160	0.7707	0.9980	0.5690
	Beefmaster	48	8	142-161	0.5887	0.9980	0.5727
	Brahman	98	8	142-161	0.7328	0.9996	0.50
	Brangus	89	21	108-161	0.8799	0.9999	0.76
TGLA 122	Holstein	50	9	128-162	0.7515	0.9994	0.5629
	Brown swiss	72	12	136-175	0.7260	1	0.6345
	Beefmaster	44	9	136-160	0.7031	1	0.6253
	Brahman	100	10	136-167	0.7900	0.9999	0.60
	Brangus	96	14	136-160	0.7176	0.9990	0.51
TGLA 227	Holstein	48	12	66-102	0.8213	0.9996	0.6601
	Simmental	56	13	76-98	0.9301	0.9999	0.8636
	Brown swiss	72	14	76-97	0.8083	1	0.7753
	Beefmaster	44	13	70-99	0.8121	1	0.7783
	Brahman	92	6	76-83	0.7414	0.9926	0.51
	Brangus	92	14	76-97	0.7764	0.9990	0.61

N = Number of observations for the estimations of allelic frequencies. PIC = Polimorphism Information Content. P.E. = Probability of Exclusion for each microsatellite

Table 3: Probabilities of exclusion of the 8 combined microsatellites in the 6 analyzed breeds

Breed					
Holstein	Simmental	Brown Swiss	Beefmaster	Brahman	Brangus
0.9988	0.9924	0.9998	0.9999	0.9930	0.9990

can lead to losses in genetic gain expected from breeding programs (Israel and Seller, 2000). These losses can be translated into a diminution of the bull`s breeding values (Banos *et al*., 2001). Some studies suggest that genetic marker information can replace that of traditional pedigree records to support animal breeding schemes (Doods *et al*., 2005).

CONCLUSION

The results obtained in this study lead us to conclude that the use of parentage verification based in a DNA test, in order to prove the accuracy of genealogical records, is justified. In the other hand, this type of analysis will also be useful in cases of legal disputes concerning the ownership of a particular animal (Liron *et al*., 2004). Furthermore, DNA microsatellite analysis is being used in paternity verification in other species, such as human (Dodds *et al*., 2005), horse (Zupanik *et al*., 2001), dogs (Tozaki *et al*., 2001; Ichikawa *et al*., 2001). Although, some studies have been

conducted to compare the usefulness of microsatellite markers with that of Single Nucleotide Polymorphisms (SNPs), most authors concluded that there is no evidence of higher exclusion probabilities when SNPs are used instead of microsatellites. This confirms the validity for this kind of tests.

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