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Research Article

Genetic variability of 10 microsatellite markers in the characterization of Brazilian Nellore cattle (*Bos indicus*)

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Abstract

We assessed the polymorphism of 10 microsatellites in Brazilian Nellore cattle (*Bos indicus*) using a commercial multiplex system. Allele frequencies, polymorphism information content, heterozygosity and exclusion probability were calculated. Allele frequencies revealed that in the sample analyzed the markers were not equally polymorphic. The exclusion probabilities and the polymorphism information content of some loci in Nellore cattle were lower than in *Bos taurus* breeds. When all the microsatellites were considered the combined exclusion probability was 0.9989. This multiplex analysis can contribute toward pedigree information, adequate genetic improvements and breeding programs.

Key words: alleles, frequencies, microsatellite, Nellore, polymorphism, zebu.

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Introduction

The *Bos indicus* Nellore herd is one of the largest commercial beef herds in the world and is well-adapted to tropical regions. According to the Brazilian Ministry of Agriculture, Livestock and Supply (http://www.agricultura.gov.br), the Nellore herd is the most important beef herd in Brazil, where the total number of both purebred and crossbreed Nellore cattle totals over 140 million head. Due to genetic improvement programs and adequate international sanitary standards, Brazil has ranked as the top beef exporter since 2003, with an export volume exceeding 1.18 million tons (Brazilian Association of Meat Export Industries http://www.abiec.com.br). Accurate pedigree information is essential to maintaining the quality of breed improvement programs and molecular markers have become an important genetic tool in animal genetics studies, allowing the analysis of genetic variability within and between herds. Many of the current molecular marker techniques are based on variations of the polymerase chain reaction (PCR) such as random amplification of polymorphic DNA (*RAPD*).

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Microsatellites markers have been widely used as a genetic markers in bovine population studies and pedigree verification (Visscher *et al*. 2002, Hansen *et al*, 2002, Ibeagha-Awemu and Erhardt, 2005), mainly because of their large polymorphism information content, widespread distribution in the eukaryotic genome (Tautz and Renz, 1984) and robust methodology. Microsatellites have been effective in evaluating differences within cattle breeds and in determining population substructures (MacHugh *et al*, 1998; Ciampolini *et al*., 1995). More than 1400 microsatellites have been mapped in the cattle genome (Luikart *et al*., 1999) and some of them have been employed in population genetics studies and parentage verification. Many microsatellite loci have been used in Nellore improvement programs but, to date, there have been no reports of pedigree verification studies using microsatellite markers, pedigree verification in Brazilian livestock currently being based on blood groups and biochemical polymorphism analyses.

The aim of the study described in this paper was to characterize Brazilian Nellore cattle through the analysis of the genetic variability of ten microsatellite markers and to evaluate if these markers are informative in parentage tests.

Materials and Methods

Sample collection and DNA extraction

We sampled 200 unrelated adult Nellore cattle (150 dams and 50 bulls) registered in their breeding associations and randomly selected from private and research herds belonging to 43 farms located in various regions of Brazil. Blood samples were collected in heparinized glass tubes and total genomic DNA isolated as described by Debomoy *et al*. (1991) and stored at -20 °C.

Microsatellite amplification

As recommended by the International Society of Animal Genetics (ISAG), ten microsatellites (Table 1) were selected for the analysis, using the Stockmarks for Cattle Bovine Genotyping Kit (Applied Biosystems Division, Perkin-Elmer, Foster City, CA). Multiplex amplification was carried out in a final volume of 15 µL containing 50 ng of template DNA, 0.5 units of AmpliTaq Gold™ polymerase (PE Applied Biosystems, Foster City, CA), 3.0 µL Stockmarks Buffer, 400 µM of each dNTP and 5.5 µL of primer mix (Table 1). The reactions were carried out using a Programmable Thermal Controller PTC-100™ (MJ Research, INC) in an initial denaturation phase of 15 min at 95 °C, followed by 31 cycles of 45 s at 94 °C, 45 s at 61 °C and 1 min at 72 °C. A final extension was carried out at 72 °C for 1 h and then at 25 °C for 2 h. After amplification, 90 µL of water was added to the tubes and 0.4 µL of this solution was mixed with 2 µL loading mix (DI formamide: dye: GS350Rox - 6:1:1) and analyzed in a 6% (w/v) denaturing gel using an ABI PRISM™ 377 DNA Sequencer. The fluorescence data was collected by GeneScan™ Analysis 2.0 and analyzed using Genotyper™ 2.0 software.

Data analysis

The GENEPOP package Version 3.4 (Raymond & Rousset, 1995) was used to calculate an exact test for deviation from Hardy-Weinberg equilibrium (HWE), allele frequencies and heterozygotic deficiency. Since the microsatellite loci have more than four alleles, an unbiased estimate of the exact HWE probability was calculated using the Markov chain method of Guo & Thompson (1992). The gene diversity (D) was calculated with FSTAT 2.9.3.2 (Goudet, 2001). Exclusion probability (EP), combined exclusion probability (CEP), expected heterozygosity (He) and observed heterozygosity (Ho), and polymorphism information content (PIC) were calculated using Cervus 2.0 software (Marshall *et al*., 1998).

Results

Ninety-four alleles were detected from the 10 loci surveyed, yielding a mean value of 9.4 alleles per locus. The allele frequencies of 10 microsatellites are listed in Table 2. Allele frequencies revealed that not all markers were equally informative. The *TGLA227*, *BM1824* and *TGLA53* loci each had one allele with a much higher frequency than the other alleles (75 bp, 180 bp and 160 bp respectively). The loci *ETH10* and *ETH3* each had two alleles with high frequencies (209 bp -207 bp and 115 bp -117 bp, respec-

Table 1 - Details of the ten microsatellite loci analyzed.

Locus	Size range (bp)	Primer sequence*	Reference
TGLA227	64-115	F: 5'-cga att cca aat ctg tta att tgc t-3' R: 5'-aca gac aga aac tca atg aaa gca-3'	Barendse *et al.*, 1992
BM2113	116-146	F: 5'-cgt gcc ttc tac caa ata ccc-3' R: 5'-ctt cct gac aga agc aac acc-3'	Bishop *et al.*, 1994
TGLA53	147-197	F: 5'-gct ttc aga aat agt ttg cat tca-3' R: 5'-atc ttc aca tga tat tac agc aga-3'	Barendse *et al.*, 1992
ETH10	198-234	F: 5'-gtt cag gac tgg ccc tgc taa ca-3' R: 5'-cct cca gcc cac ttt ctc ttc tc-3'	Toldo *et al.*, 1993
TGLA126	104-133	F: 5'-cta att tag aat gag aga ggc ttc t-3' R: 5'-ttg gtc tct att ctc tga ata ttc c-3'	Barendse *et al.*, 1992
TGLA122	130-193	F: 5'-aat cac atg gca aat aag tac ata c-3' R: 5'-aat cac atg gca aat aag tac ata c-3'	Barendse *et al.*, 1992
INRA023	193-235	F: 5'-gag tag agc tac aag ata aac ttc-3' R: 5'-taa cta cag ggt gtt aga tga act c-3'	Vaiman, *et al.*, 1992
ETH3	90-135	F: 5'-gaa cct gcc tct cct gca ttg g-3' R: 5'-act ctg cct gtg gcc aag tag g-3'	Toldo *et al.*, 1993
ETH225	135-165	F: 5'-gat cac ctt gcc act att tcc t-3' R: 5'-aca tga cag cca gct gct act-3'	Steffen *et al.*, 1993
BM1824	170-218	F: 5'-gag caa ggt gtt ttt cca atc-3' R: 5'-cat tct cca act gct tcc ttg-'3'	Bishop *et al.*, 1994

*F = Forward; R = Reverse.

Table 2 - Allele frequencies (Freq.) of the ten microsatellite loci analyzed.

Microsatellite loci																			
TGLA122		*TGLA53*		*ETH225*		*INRA023*		*BM1824*		*ETH10*		*ETH3*		*TGLA126*		*TGLA227*		*BM2113*	
Allele	Freq.	Allele	Freq.	Allele	Freq.	Allele	Freq.	Allele	Freq.	Allele	Freq.	Allele	Freq.	Allele	Freq.	Allele	Freq.	Allele	Freq.
133	0.3061	160	0.7520	138	0.0833	194	0.1757	176	0.0032	205	0.0630	101	0.0450	109	0.0026	75	0.7338	129	0.3927
135	0.0212	162	0.0160	140	0.1210	196	0.0901	178	0.1899	207	0.3445	103	0.0125	115	0.1553	77	0.1741	135	0.1723
137	0.0030	164	0.0160	142	0.0081	198	0.0135	180	0.5032	209	0.4958	115	0.3475	117	0.0211	79	0.0597	137	0.0169
139	0.0152	166	0.0200	146	0.0242	200	0.0631	182	0.2215	211	0.0420	117	0.4050	119	0.0711	81	0.0174	139	0.0621
141	0.2273	168	0.1080	150	0.0027	202	0.1261	184	0.0222	213	0.0042	119	0.1125	121	0.3000	95	0.0050	141	0.2684
143	0.0303	170	0.0200	152	0.0645	208	0.0360	186	0.0032	215	0.0210	121	0.0075	123	0.2316	97	0.0100	143	0.0876
145	0.0273	172	0.0240	154	0.0027	210	0.0135	192	0.0063	217	0.0084	123	0.0100	125	0.1447				
147	0.0606	174	0.0040	158	0.2661	212	0.0901	194	0.0285	219	0.0210	125	0.0600	127	0.0737				
149	0.0121	176	0.0080	160	0.4247	214	0.2342	196	0.0222										
151	0.0303	182	0.0080	162	0.0027	216	0.1577												
153	0.0394	184	0.0120																
155	0.0879	186	0.0080																
159	0.0030	190	0.0040																
161	0.0152																		
163	0.1182																		
165	0.0030																		

tively). The number of alleles per locus ranged from six for *TGLA227* to 16 for *TGLA122*. The *TGLA122* locus showed the highest allele polymorphism, while the *INRA023* locus displayed the highest exclusion probability. Six loci (*TGLA 53*, *ETH10*, *ETH3*, *ETH225*, *TGLA122* and *INRA023*) deviated significantly ($p < 0.05$) from HWE. A significant deficit of heterozygosity ($p < 0.01$) was detected in the *TGLA53*, *ETH10 ETH225*, *TGLA122*, and *INRA023* loci. The *ETH3* locus did not show heterozygote deficiency, although its P value was close to $p < 0.01$. The mean PIC value was 0.640 and the mean expected heterozygosity value was 0.679. Expected and observed heterozygosity, probability of exclusion and PIC values are shown in Table 3. The combined probability of parentage exclusion for the 10 microsatellites was 0.9989.

Discussion

Accurate cattle pedigree information is essential for the optimal development of breed and selection programs, improving productivity in the animal industry. Misidentification of parentage can lead to breeding inaccuracy, causing great financial losses in herd management and in the beef industry. Geldermann *et al*. (1986) estimated misidentification rates of 13% using blood group factors and biochemical polymorphisms in cattle. Ron *et al*. (1996) found a 5% misidentification rate using microsatellite analysis in Israeli dairy cattle. Rosa (1997) reported a misidentification rate of 15% in Brazilian livestock, based on restriction fragment length polymorphism (RFLP) and microsatellite analysis. Microsatellites are the most widely used molecular markers in pedigree control. The use of microsatellites

Table 3 - Variability measures of ten microsatellite markers in Brazilian Nellore cattle.

Microsatellite locus	Number of observed alleles (A)	Expected heterozygosity (He)	Observed heterozygosity (Ho)	Gene diversity (D)	Polymorphism information content (PIC)	Exclusion probability (EP)
TGLA227	6	0.428	0.368	0.428	0.390	0.230
BM2113	6	0.734	0.718	0.734	0.691	0.503
TGLA53	13	0.422	0.352	0.423	0.405	0.256
ETH10	8	0.631	0.521	0.632	0.565	0.371
TGLA126	8	0.802	0.847	0.802	0.773	0.611
TGLA122	16	0.827	0.661	0.827	0.805	0.666
INRA023	9	0.855	0.541	0.857	0.835	0.704
ETH3	8	0.698	0.630	0.699	0.646	0.445
ETH225	10	0.724	0.323	0.725	0.683	0.500
BM1824	10	0.662	0.677	0.662	0.612	0.418

with high polymorphism information content would help to correctly identify individual cattle, allowing for the better operation of cattle breeding programs.

Little information is available regarding the allele frequencies of the ten microsatellites studied in this work and the other variability estimates for Nellore cattle and, to date, there are no estimates of the multiplex variability in Brazilian cattle breeds. Since the evaluation of polymorphism is strictly dependent on the allele number and the frequency distribution of the alleles, estimates of allele frequencies are essential.

A comparison of the results obtained for *B. indicus* Nellore cattle with those of *B. taurus* breeds indicated a difference in variability for some loci, which are highly informative in *B. taurus* but less informative in *B. indicus* (Nellore). The exclusion probability values for the *TGLA227*, *ETH10* and *TGLA53* loci of taurine cattle described by Peelman *et al*. (1998) and Heyen *et al*. (1997) are much higher than that of Nellore cattle.

According to Peelman *et al*. (1998), who analyzed Belgium cattle, the number of *TGLA53* locus alleles in Holstein Friesian (13 alleles), Belgian Red Pied (12 alleles), East Flemish (12 alleles) and Belgian Blue (10 alleles) cattle were very similar to that found in Nellore cattle (13 alleles). However, we found that the exclusion probability for the *TGLA53* locus in Brazilian Nellore (EP = 0.256) cattle is much lower than in the four Belgian breeds (Holstein Friesian = 0.742, Belgian Red Pied = 0.711, East Flemish = 0.698 and Belgian Blue = 0.682). We obtained similar results for the *TGLA227* locus (EP 0.230), much lower than the values described by Heyen (1997) for Holstein (0.69), Red Angus (0.63) and Gelbvieh (0.68) cattle. Thus, the effectiveness of these markers in European *B. taurus* cattle is not always the same for Indian *B. indicus* zebu (Brahman, Nellore) cattle. The substitution of the markers with low variability values for others with improved EP values could render this multiplex more efficient for pedigree verification and individual identification in Nellore. Characterization of Brazilian cattle breeds with microsatellite loci is useful to identify informative markers for each breed, optimizing parentage tests along with the variability values of each marker, thus using the least number of markers with higher levels of information while simultaneously facilitating genotypic identification.

The combined exclusion probability value for the 10 loci was 0.9989, an acceptable value more than ideal for parentage tests (Baron *et al*, 2002). Jia *et al* (2004) showed that the CEP value was 0.9957 for Holstein Friesian cattle using six microsatellite markers, while Radko *et al* (2002) obtained a CEP value of 0.9999 using 11 microsatellites and it is known that the CEP values found in Nellore cattle is lower than that of other taurine breeds (Heyen *et al*., 1997).

In our study we found significant ($p < 0.01$) deviations from HWE for six loci (*TGLA122*, *INRA023*, *TGLA53*, *ETH10*, *ETH225* and *ETH3*). Machado *et al* (2003) also found significant deviations from HWE for Nellore, Gyr and Guzerat cattle breeds using microsatellites markers. Almeida *et al* (2000) found that the *TGLA122* locus was in HWE in Brazilian hybrid bovine breed (5/8 Aberdeen Angus x 3/8 Nellore). We found deviations from HWE caused by heterozygote deficiency at the *TGLA122*, *INRA023*, *TGLA53*, *ETH10* and *ETH225* loci. Beja *et al*. (2003) and Loftus *et al*. (1999) found deviations from HWE in other European bovine populations, also caused by a heterozygosity deficit, and similar results have been reported by Loftus (1999) in six populations, including Indian Nellore cattle.

Several factors can lead to heterozygote deficiency, including null alleles, assortive mating, the Wahlund effect, selection against heterozygotes, inbreeding, or a combination of these. Null alleles are alleles that are not amplified (usually due to a mutation in one of the primer binding sites) and are commonly reported in microsatellite studies as being the source of heterozygosity deficit (Pemberton *et al*., 1995). The frequency of microsatellite loci containing null alleles has proved to be as high as 30% in humans (Callen *et al*., 1993). In paternity tests, an undetected null allele may have profound consequences, since it may cause rejection of an otherwise correctly assigned parent (Holm *et al*., 2001).

To date, there are no reports of studies on Nellore cattle indicating the presence of null alleles for the markers analyzed, although the presence of null alleles has previously been observed in segregation analyses using other microsatellite loci in Nellore cattle (Tambasco *et al*., 2000). This hypothesis cannot be excluded because segregation analysis using the loci evaluated in this study has not yet been undertaken for Nellore cattle.

Despite the paucity of information provided by some of the loci analyzed in this study, the use of this multiplex analysis proved efficient in Nellore characterization and can be used in pedigree verification.

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