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Research Article

Validation of a microsatellite panel for parentage testing of locally adapted and commercial goats in Brazil

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Abstract

Brazilian goats are generally kept in small herds and extensive rearing systems, mainly in the northeastern region of the country. Despite production improvement in recent years, the lack of pedigree control has affected genetic progress. This study aimed to validate a panel of 16 microsatellites for parentage testing in locally adapted and commercial goats breeds raised in Brazil, as well as to compare its efficiency with the panel recommended by the Brazilian Ministry of Agriculture, Livestock and Supplies (MAPA) in 2004. The number of alleles and expected heterozygosity (He) per marker ranged from four to 18, and from 0.051 to 0.831, respectively. Using all markers, 100% of parentage cases of the validation dataset were resolved with a strict confidence level of 95%. The 16 microsatellites panel showed adequate exclusion power (99.99%) and identity accuracy (99.99%). Suggestions for improvement of the marker panel endorsed by MAPA are provided.

Key words: parentage errors, animal breeding, animal genetic resources, *Capra hircus*.

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Introduction

Goats are one of the most important livestock species in the world, mostly because of their meat and milk production. According to IBGE (2010), the Brazilian goat population was estimated at 9.31 million heads, with 90% of the animals being raised in the northeastern region of the country. In recent years, there have been increases in herd size and productivity (Lopes *et al.*, 2012), but management practices still face many challenges, particularly with pedigree record keeping, which is of fundamental importance for adequate operation of production farms and genetic improvement programs.

Correct pedigree information is essential for performing genetic evaluations, as errors lead to incorrect estimates and low accuracies of estimated breeding values (see Maichomo *et al.*, 2008). Pedigree errors of about 10% may lead to reductions in selection response of two to three percent in dairy cattle (Visscher *et al.*, 2002), while different studies have reported observed pedigree errors of up to 23% in cattle in several countries (Christensen *et al.*, 1982; Ron *et al.*, 1996; Banos *et al.*, 2001; Weller *et al.*, 2004; Jiménez-Gamero *et al.*, 2006).

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).

In Brazil, estimated pedigree errors of more than 25% in Gir cattle have been observed (Baron *et al.*, 2002), while in sheep these have reached 15.5% (Barnett *et al.*, 1999).

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Due to this, the Brazilian Ministry of Agriculture Livestock and Supply (MAPA) issued in 2004 norms requiring DNA testing for herdbook registration of livestock, along with accreditation instructions for laboratories performing animal genetic identification with DNA fingerprinting methods. Eight microsatellite markers (OarCP49; OarFCB11; OarAE129; OarFCB304; MAF214; OMHC1; SPS0113; D5S2) were listed at the time as required for both sheep and goat genotyping. Souza *et al.* (2012) evaluated the efficiency of this panel in a sample of Santa Inês hair sheep and obtained lower combined probabilities of exclusion (PEC) than with other panels proposed by the authors.

Araújo *et al.* (2010) validated a panel of 11 microsatellite markers for paternity testing of Brazilian goats, with combined probabilities of exclusion (PEC) of paternity of 0.999591 and 0.988375, in cases where the maternal genotype was known or unknown, respectively. When used to evaluate a group of registered goats, this particular set of markers detected 10% of paternity errors. Although this was a recent study, none of the markers used are present in the list sanctioned by MAPA in 2004.

The present study was performed to evaluate the efficiency of a panel of 16 microsatellite markers, including the eight recommended by MAPA (2004), in parentage testing of Brazilian goats from four commercial and four naturalized breeds. This study is part of the Brazilian Dairy Goat Breeding Plan (DGBP), an initiative coordinated by Embrapa Goat and Sheep, and has as partners universities and the Association of Goat and Sheep Breeders of Minas Gerais State (CAPRILEITE/ACCOMIG). The main objective of this plan is to structure a community based dairy goat national databank and conduct progeny tests for the main dairy goat breeds raised in the country (Facó *et al.*, 2011; Lôbo *et al.*, 2010).

Materials and Methods

A total of 120 samples of genomic DNA from locally adapted goat breeds: Canindé (CA, N = 16), Marota (MA, N = 23), Moxotó (MO, N = 22) and Repartida (RE, N = 16); as well as samples from commercial breeds: Saanen (SA, N = 17); Alpine (AL, N = 06), Anglo Nubian (AN, N = 04) and Mambrina (MB, N = 16); were used. Of these, 102 samples were derived from the DNA and tissue Gene Bank maintained by Embrapa Recursos Genéticos e Biotecnologia and were used to create the allele frequency databank (training dataset) for obtaining the parentage estimates. The remaining 18 samples were obtained from the Association of Goat and Sheep Breeders of Minas Gerais (CAPRILEITE/ACCOMIG). A total of six known trios from the Saanen (N = 4) and Alpine (N = 2) breeds were also included. The trios were formed each by a buck, doe and kid, and the trios were independently sampled throughout the farms covered by ACOOMG. Genomic DNA was extracted using an adapted protocol described by Miller *et al.* (1989).

A total of 16 microsatellites were used in the study: eight markers were recommended by MAPA (MAPA, 2004), five were derived from the FAO/ISAG panel (ISAG, 2010) for goat parentage testing, and three were derived from the FAO (2011) panel recommended for studies with genetic diversity in sheep and goats (Table 1). Amplification of the markers was carried out using the Master Mix Kit for PCR-multiplex (Qiagen), following manufacturer's recommendations, using 4.5 ng genomic DNA and 0.05-0.15 μM of each primer, at a final volume of 5 μL. Amplification conditions were: 95 °C for 15 min, 35 cycles at 95 °C for 5 min, 57 °C for 90 s and 72 °C for 1 min, followed by a final extension step of 72 °C for 30 min. Information regarding expected allele sizes for each marker, fluorescent label, multiplex and type of marker in the MAPA-2004 and complementary panels (PC) are presented in Table 1.

Amplified fragments were separated in an automated sequencer (ABI Prism 3100, Applied Biosystems), and generated data was analyzed with GeneScan v.3.1 and Genotyper v.3.7.0.1 (Applied Biosystems) software for allele and genotype calling. Allelic class determination was carried out using FlexBin v.2.0 software (Amos *et al.*, 2006).

Cervus v.3.0.3 software (Marshall *et al.*, 1998) was used to obtain estimates of allele number (Na), observed (Ho) and expected (He) heterozygosity (Nei, 1978), polymorphism information content (PIC) (Botstein *et al.*, 1980), and frequency of null alleles (FAN) for each marker.

Table 1 - Marker code, expected allele size, type of fluorescence and multiplex.

Marker	Alleles (pb)	Fluorescence	Multiplex
ILSTS011[2]	250-300	6-FAM	A
ILSTS087[2, 3]	135-155	6-FAM	A
OMHC1[1, 2]	180-208	6-FAM	A
TCRVB6[2]	217-255	NED	B
INRA05[2, 3]	135-149	6-FAM	B
INRA63[2, 3]	164-186	6-FAM	C
SPS0113[1, 2]	134-158	TET	C
SRCRSP5[2]	99-135	6-FAM	C
MCM527[2]	165-187	HEX	D
INRABERN172[2, 3]	234-256	6-FAM	D
OarFCB11[1, 2, 3]	122-140	6-FAM	E
D5S2[1, 2]	190-204	6-FAM	E
OarCP49[1, 2]	80-100	6-FAM	F
MAF214[1, 2]	181-265	HEX	F
OarAE129[1, 2]	135-165	HEX	*
OarFCB304[1, 2]	150-188	NED	*

1 - Panel recommended by MAPA (2004); 2 - Complementary Panel used in this study (PC); 3 - FAO/ISAG panel (2011). *Markers amplified individually.

The probability of exclusion considering only offspring and probable sire (PE1) and a known parent (PE2), and the probability of identity (PI) were estimated for each marker and for three distinct marker panels: Panel 1 - all 16 markers; Panel 2 - eight markers recommended by MAPA (2004); and Panel 3 - seven markers with highest PIC and PI (ILSTS87; OMHC1; TCRVB6; MCM527; INRA172; OarFCB11; OarAE129).

An exact test using a Markov chain implemented in Genepop software (Raymond and Rousset, 1995) was used to test for Hardy-Weinberg equilibrium (HWE) at each marker (Guo and Thompson, 1992). The Δ test (Delta) in Cervus (Marshall *et al.*, 1998) was used to estimate the confidence of informed paternity. Two simulations for each panel were carried out for correct identification of the probable sire: (1) identification of sire without dam information, and (2) no parental information. In the simulations, 10,000 progeny were used considering the same number of male and female candidates (n = 5) and with 100% of candidate parents sampled. The proportion and minimum quantity of markers genotyped were 91% and 10 markers, respectively, when considering the full panel. For the reduced panels, the minimum number of markers genotyped was six (MAPA, 2004) and five (most informative markers), respectively. The genotyping error was set at 1%, and strict and relaxed confidence levels were specified as 95% and 80%, respectively. A paternity test was carried out wherein the most probable sire was confirmed based on LOD scores greater than zero and the true sire presenting the highest LOD score.

Results

All 16 markers amplified polymorphic fragments in the eight tested breeds (Table 2) of the training dataset. In some breeds, the markers SPS0113 (Alpine), OarCP49 (Moxotó, Anglo Nubian, Marota and Mambrina), ILSTS11 (Repartida), and D5S2 (Marota and Mambrina) showed amplification problems, generating outlier allelic patterns. To avoid genotyping errors, some genotypes of the referred markers were excluded from further statistical analysis.

The number of alleles varied from four (SPS0113, D5S2 and OarCP49) to 18 (OarFCB11). D5S2 presented the lowest values for all parameters analyzed and was the only marker which remained in HWE ($p > 0.05$), while all other markers showed significant deviations from HWE ($p < 0.05$). The highest expected heterozygosity (He) was found for markers TCRVB6 (0.83) and OMHC1 (0.83). Three markers were found to be the most informative, with highest probabilities of exclusion and identity, and PIC (OarFCB11, OMHC1, and TCRVB6). Conversely, D5S2 and OarCP49 showed the lowest information content.

Panel 3 showed the highest number of alleles (10.43), Ho (0.66), He (0.80), PIC (0.77) and PIT (87.26%). Although Panel 1 showed a lower estimated mean number of alleles, higher Ho, He, and PIC estimates were observed in comparison with Panel 2. Estimated PEC1 and PEC2 were higher for Panel 1 than for Panels 2 and 3 (Table 3).

In general it was observed that locally adapted Brazilian breeds showed higher values than commercial breeds for every genetic index used (Table 4). For the probability

Table 2 - Combined genetic variability parameters for each microsatellite marker analysed in eight goats breeds.

Marker	Na	Ho	He	PIC	PE1	PE2	PI	HWE	FAN
ILSTS011	7	0.52	0.71	0.67	0.30	0.49	0.88	0.0024**	+0.14
ILSTS087	7	0.68	0.77	0.73	0.38	0.56	0.91	0.00001***	+0.06
OMHC1	10	0.83	0.83	0.81	0.5	0.67	0.95	0.00001***	-0.01
TCRVB6	11	0.70	0.83	0.80	0.49	0.66	0.95	0.0009***	+0.08
INRA05	5	0.88	0.63	0.56	0.21	0.36	0.79	0.00001***	-0.19
INRA63	7	0.56	0.68	0.63	0.27	0.44	0.85	0.00001***	+0.09
SPS0113	4	0.50	0.58	0.53	0.18	0.35	0.78	0.00001***	+0.08
SRCRSP5	8	0.86	0.72	0.67	0.31	0.48	0.88	0.0013***	-0.09
MCM527	7	0.71	0.76	0.73	0.37	0.56	0.91	0.00001***	+0.03
INRABERN172	6	0.62	0.74	0.70	0.34	0.51	0.89	0.00001***	+0.08
D5S2	4	0.05	0.05	0.05	0.09	0.03	0.10	1.0000^{ns}	-0.01
OarCP49	4	0.94	0.51	0.39	0.13	0.20	0.64	0.00001***	-0.30
MAF214	10	0.86	0.70	0.66	0.30	0.49	0.87	0.00001***	-0.16
OarFCB11	18	0.61	0.82	0.80	0.5	0.67	0.95	0.00001***	+0.14
OarAE129	8	0.61	0.78	0.75	0.40	0.58	0.92	0.00001***	+0.13
OarFCB304	12	0.51	0.81	0.79	0.48	0.65	0.95	0.00001***	+0.23

Na = number of alleles; Ho = observed heterozygosity; He = expected heterozygosity; PIC = Polymorphism information content; PE1 = probability of exclusion 1; PE2 = Probability of exclusion 2; HWE = Hardy-Weinberg equilibrium; ** $p < 0.001$; ***$p < 0.001$; FAN - Frequency of Null Alleles.

Table 3 - Genetic variability parameters estimated for three different microsatellite panels used in goats sampled in Brazil.

Panel	Number of markers	Nam	Ho	He	PIC	PEC1	PEC2	PI %	PIT %
1	16	8.00	0.66	0.68	0.64	0.99	> 0.9999	> 99	85.99
2 (MAPA 2004)	8	8.75	0.62	0.64	0.6	0.96	0.9958	> 99	85.83
3	7	10.43	0.67	0.80	0.77	0.98	0.9989	> 99	87.26

Nam = mean number of alleles; Ho = observed heterozygosity; He = expected heterozygosity; PIC = polymorphic information content; PEC1 = combined probability of exclusion 1; PEC2 = combined probability of exclusion 2; PI = probability of identity; PIT = proportion of genotyped individuals.

Table 4 - Genetic variability parameters estimates per breed using a panel of 16 microsatellite markers.

Breeds		Parameters						
		Nam	Ho	He	PIC	PEC1	PEC2	PI
Commercial	AL	2.94	0.67	0.56	0.45	0.9518	0.9964	> 0.9999
	SA	4.81	0.63	0.61	0.54	0.9855	0.9996	> 0.9999
	MB	4.69	0.70	0.65	0.58	0.9913	0.9999	> 0.9999
	AN	3.06	0.68	0.67	0.48	0.9712	0.9983	> 0.9999
	Mean	3.88	0.67	0.63	0.51	0.9750	0.9986	> 0.9999
Brazilian	CA	4.88	0.68	0.65	0.58	0.9923	0.9999	> 0.9999
	MO	5.25	0.63	0.62	0.56	0.9899	0.9998	> 0.9999
	MA	4.94	0.64	0.61	0.54	0.9843	0.9996	> 0.9999
	RE	5.19	0.66	0.65	0.57	0.9924	0.9999	> 0.9999
	Mean	5.07	0.65	0.63	0.56	0.9897	0.9998	> 0.9999

Nam = mean number of alleles; Ho = observed heterozygosity; He = expected heterozygosity; PIC = polymorphism information content; PEC1 = combined probability of exclusion 1; PEC2 = combined probability of exclusion 2; PI = probability of identity; Alpine (AL); Saanen (SA); Mambrina (MB); Anglo Nubian (AN); Canindé (CA); Moxotó (MO); Marota (MA); and Repartida (RE).

of identity, commercial breeds showed values higher than 99.99%. Brazilian Caninde (CA) and Repartida (RE) breeds showed values higher than the mean for He, PIC, for He, PIC, as well as PEC1 and 2 (probability of exclusion 1 and probability of exclusion 2), while the Moxoto (MO) breed showed the highest number of alleles (5.25).

For the parentage test validation it was possible to obtain results from five of the six trios analyzed, and in 100% of the cases the correct father was assigned for each of the five trios with a strict level of confidence (95%), and with either Panel 1 or 2. Panel 3 could only be used to solve 80% of the cases (four trios) at a 95% restricted confidence level. No incompatibilities between genotypes of all five trios were observed with Panel 2, while for the other two panels, the marker OarFCB304 showed small inconsistencies in two trios. Nevertheless, these issues did not significantly affect the combined exclusion power of the panels (Table 3).

Discussion

Goat parentage verification tests are becoming routine in Brazil as the sector is experiencing a production growth and a re-organization of the main actors of the supply chain (farmers, government and breed associations). The microsatellite panel sanctioned by MAPA in 2004 for sheep and goat parentage verification in Brazil was based on available literature at the time (Luikart *et al.*, 1999; Arranz *et al.*, 2001; Crawford *et al.*, 2000; Farid *et al.*, 2000; Stahlberger-Saitbekova *et al.*, 2001; Tomasco *et al.*, 2002; Rychlik *et al.*, 2003) and did not consider updates developed by the International Society of Animal Genetics and the genetic diversity of Brazilian breeds. Souza *et al.* (2012) evaluated this panel in Santa Inês sheep and found that some of the used markers were not very informative due to the low number of observed alleles, PIC, and consequently, the low individual and combined probability of exclusion of the markers in the panel. Markers SPS0113, D5S2 and OarCP49, which are part of the MAPA 2004 recommended panel, showed the lowest numbers of observed alleles in the present study (Table 2), and therefore should be replaced by more informative markers.

The lowest number of alleles (Na = 4) was observed for markers D5S2, SPS0113 and OarCP49, which were all part of the MAPA 2004 recommended panel. Markers ILSTS11, ILSTS87, TCRVB6, INRA63, INRABERN172, SPS0113, OarFCB11, OarAE129 and OarFCB304, five of which are included in the MAPA 2004 panel, showed FAN greater than 0.05 (Table 2) and, according to Marshall *et al.* (1998), should not be used for paternity testing as they tend to have reduced heterozygosity. High frequencies of null alleles lead to high rates of genotyping errors of heterozy-

gotes, resulting in incorrect exclusions of dam-offspring or sire-offspring pairs.

Heterozygosity estimates were high for most of the tested markers (Luikart *et al.*, 1999; Menezes *et al.*, 2006; Carolino *et al.*, 2009; Araújo *et al.*, 2010; Zhang *et al.*, 2010). Panel 3 showed a He of 80%, while in Panels 1 and 2 observed values were close to 70% (Table 3). The lowest heterozygosity estimates (Ho and He) were seen for D5S2 (0.053 and 0.054), which remained in a state of HWE, while the other markers showed differences in observed and expected genotype frequencies that led to significant HWE deviations ($p < 0.05$; Table 2). These deviations may be due to matings of closely related animals, as well as other unknown population sub-structuring.

The mean PIC value was highest for Panel 3 (0.77), which also showed the highest mean number of observed alleles (10.43, Table 3). As the PIC value is totally dependent on microsatellite frequencies this should not be the only parameter used for selection or exclusion of a marker for use in a panel for genetic analysis (Moazami-Goudarzi *et al.*, 1994).

The effectiveness of the panel was also analyzed by the probability of exclusion (PE) which is a parameter widely used for verification of pedigree (Araújo *et al.*, 2010; Stevanovic *et al.*, 2010; Zhang *et al.*, 2010; Adamov *et al.*, 2011; Saberivand *et al.*, 2011; Souza *et al.*, 2012). The analysis for the panel of 16 markers confirmed paternity with PEC1 and PEC2 equal to 99.98% and 99.99%, respectively (Table 3). In the other evaluated panels, PEC1 and PEC2 were less than 99.98%, confirming exclusion probabilities obtained by Souza *et al.* (2012) for Santa Inês sheep (99.708% and 99.799 for PEC1 and PEC2, respectively) using the MAPA 2004 panel, and above 99.99% for both probabilities when the number of markers was increased to 23.

In commercial goat breeds, the lower values obtained for the parameters studied (Nam, He, PIC, PEC1 and PEC2) may be the result of selection pressure that resulted in a loss of genetic diversity when compared with these parameters in Brazilian local adapted goat breeds, as well the low number of founder animals analyzed. Among specialized goat breeds, only the Mambrina (MB) showed optimal PEC2 (99.99%), while for all Brazilian goat breeds PEC2 was above 99.9% (Table 4). Luikart *et al.* (1999) found that the probability of exclusion reached 99.99% for Saanen (SA). Araújo *et al.* (2010) observed an exclusion probability greater than 99.99% with 11 markers in three goat breeds (Saanen, Alpine and Moxotó).

The obtained probability of identification (PI) estimates were > 99.99% in all studied breeds. Therefore, the three panels may be useful for identification of any individual belonging to these breeds (Table 4). However, to minimize costs and time, markers with the lowest probabilities of identity (D5S2 and OarCP49) should be excluded from further studies.

Panel 1 showed adequate paternity exclusion power in the evaluated goat breeds and could be used efficiently to verify and estimate parentage error rates in herds included in the National Dairy Goat Genetic Evaluation and Breeding programs led by Embrapa. In addition, any of the three evaluated panels could be efficiently used for individual identification, as all three panels showed accuracy above 99.9%.

In the second semester of 2012, MAPA published a new list of 17 microsatellites (MAPA, 2012) from which a minimum of eleven markers should be used for parentage testing. This new panel maintained three markers used in the original panel and contains five markers from the FAO diversity (2011) or ISAG paternity (2011) panels. Three of the markers in the full panel studied here (OarFCB11, ILSTS087 and MCM527) are included in this new MAPA panel. However, three other makers (OMHC1, OarE129 and OarFCB304), which were part of the original MAPA 2004 panel, have been removed from the newer list. Markers included in the MAPA 2012 panel, such as SRCRSP5, INRABERN172 and INRA63 presented low PIC (< 0.7) and PI (< 0.9) in the present study, which corroborates results reported by Araújo *et al.* (2010). Markers (OarCP49 and D5S2) indicate in our study to be highly informative in the tested breeds, were removed from the new panel (MAPA 2012), while other markers found to be less informative (SRCRSP5, ILSTS005, INRABERN172 and INRA63) were maintained. Changes in established parentage verification panels can lead to major financial impacts for farmers, as reproductively active animals that have been genotyped with the old panel have to be re-tested with the new additional markers. McClure *et al.* (2012) addressed these issues, emphasizing that new genotyping requirements can face major limitations, especially when considering historic animals without a viable DNA source due to culling, death, or change in ownership of the animal.

Recent advances in the use of genomic technologies are profoundly impacting several livestock industries around the world. The widespread use of low-cost high density SNP marker panels in routine genetic evaluations and breeding programs are driving a paradigm shift to a new structure in which microsatellite marker data is no longer needed for paternity testing. Studies validating imputation methods to transpose microsatellite data from historical animals to SNPs contained in commercial panels now routinely used for testing registered cattle and sheep have been reported (McClure *et al.*, 2012). Although this transition should be slower for goats, it can be expected that these new technologies should be fully embraced within less than ten years.

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