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Evaluation of nine microsatellite loci and misidentification paternity frequency in a population of Gyr breed bovines

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Avaliação de nove locos microssatélite e freqüência de paternidade incorreta em uma população de bovinos da raça Gir

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SUMMARY

Paternity misidentification is harmful due to the reduction in annual genetic earnings of the population and because it endangers an efficient genetic improvement program. The objectives of the present study was to evaluate nine microsatellites in Paternity Testing and to investigate misidentification paternity frequency in families of Gyr breed bovines population. In the present experiment blood samples from forty Gir breed families (bull / cow / calf), registered pure breed in the Zebu Breeders Brazilian Association (ABCZ) were used. The most part of the microsatellites used in this work were recommended by the International Society of Animal Genetics (ISAG). The genomic DNA extraction was performed from whole blood samples. The microsatellites TGLA122, TGLA126, BM1824, BMS2533, SPS115, ETH3, ETH10, ETH225 and POTCHA were amplified by PCR. The amplification products were separated by electrophoresis in denaturing polyacrylamide gel. From the obtained data, allele frequencies, Gene Diversity, Polymorphism Informative Content and Probability of Exclusion for each microsatellite marker were calculated. The genotype frequencies, Heterozygosity, Combined Probability of Exclusion and Probability of Paternity have also been calculated in the considered families. The Combined Exclusion Probability for all microsatellites was around 0.9789. The Paternity Testing results showed misidentification in eleven of the 40 studied families, that means, 27.5% of the sample. The Paternity Probability ranged from 0.8691 to 0.9999, and the mean was 0.9512.

PALAVRAS-CHAVE: DNA. Microsatellites. Paternity. Bovine. Gyr.

INTRODUCTION

Gyr breed is essential to *Girolando* cattle formation (5/8 Holstein + 3/8 Gyr). Both Gyr and *Girolando* are phenotypically superior, much more adapted to climate and economical traits in Brazil and with crucial qualities for a good milk production on the tropics. *Girolando* cattle is treated by the hybrid strenght showing rusticity and adaptation to the tropics peculiar to Gyr breed and good milk production trait from Holstein breed.

The right kinship between members of a population is prerequisite for an efficient genetic improvement program[22, 8, 11].The estimation of population genetic standards and individual genetic merit through an animal pattern depends on genealogy once these patterns use performance data from kinship animals[17] . A small misidentification percentage excessively endangers genetic patterns estimation[15] . In despite of this, several farms employ management practices that endanger the information related to genealogy.

Geldermann et al.[8] suggested a decrease between 8.7% and 16.9% on cattle genetic profit yearly, for a 15% misidentification frequency. Ron *et al*.[17] suggested a 5% increase to yearly genetic profit when Paternity Test is performed on bulls sibs tested yearly in Israel.

Bovine cattle paternity studies, using blood type, proteins and molecular markers showed a high frequency of incorrect paternity in Israel (5%), Germany (4-23%), Denmark (8-30%) and Ireland (20%) explaining its use in genetic improvement programs [8, 3, 17].

Rosa[18] performed Paternity Testing by molecular markers in Nelore bovine families. Results showed paternity misidentification in 15% of the studied families which justifies the use of these tests on genetic improvement tests.

Parental relations between individuals may be proved using several genetic markers categories, resulting on Paternity Testing. Paternity biological information consists on genetic inheritance that a sib inherited from a mother and a prospective father. Once the inherited genetic part from the mother is screened, it is necessary to investigate if the rest of the information is transmitted from the prospective father. If the latter possesses hereditarian characteristics

transmitted to the sib, it can not be excluded from paternity and the result is shown in paternity probability. If, on the other hand, the prospective father does not have these traits, he is excluded from paternity possibility.

At first, polymorphism of morphological markers and of blood types, biochemical polymorphisms generated by Major Hystocompatibility Complex (MHC) were used for this purpose[18]. Therefore, these markers categories do not give conclusive results, and due to the great number of genetic systems need for an adequate final result, Paternity Testing once done, has its use limited due to costs.

Recent advancements on molecular biology such as Polymerase Chain Reaction (PCR) development[16] and the constant discovery of new molecular markers are significantly helping solve these limitations. Molecular markers which can be highlighted as the most apropriate ones for Paternity Tests are: Restriction Fragments of Length Polymorphism (RFLPs) and multilocus minisatellites which allows an individual pattern of bands known as DNA-fingerprinting and mainly specific microsatellites loci. Microsatellites are generally highly polymorphics (great number of alleles in a locus) even in endogamic populations and also with the advantage of being codominants. They are highly frequent, well distributed along the genome and easily amplified by PCR. These traits contribute to determine, with a higher reliance, paternal and maternal origin of each allele from microsatellite expressed in progeny. Microsatellites are being used aiming to identify paternity in several domestic animals species like bovines [11, 21, 23] ,swine [12], canines [7] and caprines[1].

The objectives of the present study were to investigate misidentification paternity frequency in a population of Gyr breed animals using DNA microsatellites and the evaluation of potential use of these microsatellites in Paternity Tests and individual identification in Gyr breed bovines.

MATERIAL AND METHOD

The experiment was conducted at BIOGEM (Laboratory of Biotechnology and Molecular Genetics) of the Department of Genetics, Institute of Biosciences, São Paulo State University (UNESP), campus of Botucatu. For experimental analysis blood samples from forty Gyr breed families (bull/cow/calf) registered pure breed in the Zebu Breeders Brazilian Association (ABCZ) were used. The families were sampled in a way to guarantee proportionality using bulls on the examined herd, i.e., bulls which were more used had a greater representative family in the sample.

Seven of nine microsatellites used on this work are recommended for bovine paternity tests by the International Society of Animal Genetics[13] (ISAG) based on criteria established by Food and Agriculture Organization (FAO).

Total blood samples (5ml) were collected using vacutainer tubes with 7.5mg EDTA. The blood was homogenized in EDTA and kept frozen on ice. After being collected the blood was kept in a refrigerator at 4°C until DNA extraction. For genomic DNA extraction a Genomic Prep™ Blood DNA Isolation Kit (AMERSHAM PHARMACIA) was used. DNA extraction was done from 300ìl whole blood.

After quantification and dilution of DNA samples, DNA regarded regions were amplified by PCR technique. Each reaction was done with a 25ìl final volume and amplification mixture was: 50 ng genomic DNA from whole blood leukocytes; 0.16 ìM from each primer; 10 mM Tris-HCl pH 8.0; 50 mM KCl; 2.0 mM $MgCl_2$; 0.2mM of each dNTP and 1U Taq DNA polymerase. Amplification reactions were done in a M.J. Research, PTC 100 Model thermocycler and the following 5 steps were: (1) initial denaturation of double band at 94°C for 3 minutes, (2) denaturation at 94ºC for 1 minute, (3) primers annealing between 54°C and 60ºC depending on their constitution for 30 seconds, (4) extension at 72°C for 1 minute and (5) final extension 72ºC for 3 minutes. Steps 2,3 and 4 constitute a cycle that was repeated 32 times. After the last cycle being completed by step 4 and final extension occurred the temperature fell down and was kept at 4ºC (cooling) only to preserve the products .

The Tab. 1 presents primers pairs used for amplification, annealing temperature (AT) and reference of each microsatellite studied.

The gel for vertical electrophoresis separation of amplified DNA fragments was done in sequencing glass plates (36.4 cm x 19.6 cm), due to the need of a migration distance of at least 30 cm, for a perfect separation of different alleles allowing estimation and identification of its length in base pairs (pb). A 6% denaturated polyacrylamide gel was used, to permit a good fragment separation of different sizes.

A 20 ul sample with denaturing loading buffer and amplified DNA 1:1 proportion was applied in the gel after denaturation at 95ºC for 2 minutes. A constant potency of 40 W was applied for the necessary period (2 to 4 hours depending on the average size of alleles) for fragments migration. A molecular weight pattern with a 10 pair basis intervals fragments (10 bp DNA Ladder) from GIBCO BRL company was added in two lanes of each gel. From comparing the migration distances of the bands and the standard ones it was possible to determine DNA fragment sizes of each individual for each microsatellite.

After electrophoresis, DNA fragments (bands) were detected by silver nitrate staining. The amplified fragments were visualized under white light and photographed in 667 Polaroid film (Fig. 1).

Lanes 1,2,3,5....17,19 and 20 DNA of the animals studied. Lanes 4 and 18, DNA ladder 10 pb. The numbers on the left side of the figure indicate DNA fragments size in base pairs.

From these data were calculated allelic frequencies and Gene Diversity (GD)[24], Polymorphism Informative Content (PIC)[5] and Probability of Exclusion (PE)[6] for each microsatellite marker. Combined Probability of Exclusion (CPE)[17] was calculated for microsatellites group used. It were also calculated, genotypic frequencies and heterozigosity (Het)[24]. Probability of Paternity (PP) estimative[10] was performed in all the families where there was not paternity exclusion. Hardy-Weinberg equilibrium was tested for each marker locus.

RESULTS AND DISCUSSION

Paternity Testing effectiveness do not depend upon the number used microsatellites but on the level of informativeness that these markers provide. The level of informativeness of a microsatellite is determined by its values of Polimorphism Informative Content (PIC), Heterozigosity (Het), Gene Diversity (GD) and Probability of Exclusion (PE) and these values are dependent on the number of alleles and on the frequency distribution of these alleles on the population.

The results of the microsatellites markers potential use in paternity tests and on the control of individual identification on the studied population are showed on Table 2.

PIC, GD, Het and PE results for BMS2533 and TGLA122 microsatellites indicate the high level of informativeness of these markers on the studied samples in terms of highly variability found. So, these microsatellites showed to be adequate to perform a Paternity Testing and for the individual characterization on the samples population of Gyr breed. The same is not true for ETH10, SPS115, TGLA126, ETH3, POTCHA, BM1824 and ETH225 markers.

Microsatellites like ETH3, ETH225 and BM1824, recommended by ISAG for Paternity Tests in bovines, showed low PE. On the other hand, BMS2533 microsatellite not commonly used for these aims showed high Exclusion Probability. This fact makes clear the need of characterization for different populations or lineages within a breed in which one wants to perform a Paternity Testing, since the number of alleles and allelic frequencies can be different in different populations of the same breed.

The Table 3 shows Combined Probability of Exclusion (CPE) increase in terms of the microsatellites numbers used.

Combined Probability of Exclusion (CPE) obtained with the use of 9 microsatellites (0.98) was smaller than the optimal value (0.99). Combined Exclusion Probability for 7 markers with PE near to 0.5 would be 0.992. A CPE of same magnification (0.991) would be reached with only 4 markers with PE near to 0.7.

The frequency of paternity misidentification found in this study was 27.5% (11 in 40). Baron et. al [2] used microsatellites markers to find 36% of paternity misidentification in Gir Breed families of bulls submitted on a progeny tests. These values are above the values obtained by other authors, studying other races in another countries, mentioned by Ron *et al.*[17]. Misidentification Paternity frequency found in this study may reflect brazilian reality, and proves that a more efficient control over genealogical

Table 1

Microsatellites (MS) markers used, localization chromosome, primers sequences, annealing temperature and references of microsatellites studied. Botucatu – SP, 1999.

MS	Chrom.	*primers*	AT °C	Reference
ETH225	9	5'- GATCACCTTGCCACTATTTCCT- 3'		
		3'- ACATGACAGCCAGCTGCTACT- 5'	60	19
ETH10	5	5'- GTTCAGGACTGGCCCTGCTAACA- 3'		
		3'- CCTCCAGCCCACTTTCTCTTCTC- 5'	58	20
ETH3	19	5'- GAACCTGCCTCTCCTGCATTGG- 3'		
		3'- ACTCTGCCTGTGGCCAAGTAGG- 5'	60	20
BMS2533	15	5'- TGAAGTAAGTAAGCACACAAGCA- 3'		
		3'- TTGATCATCTTTAGGTCCATCC- 5'	56	14
BM1824	1	5'- GAGCAAGGTGTTTTTCCAATC- 3'		
		3'- CATTCTCCAACTGCTTCCTTG- 5'	58	4
POTCHA	15	5'- GTAAACACAGTTCCCTGGAGAG- 3'		
		3'- ATGCCAACTTTTCCCATCAC- 5'	60	14
TGLA126	20	5'- CTAATTTAGAATGAGAGAGGCTTCT- 3'		
		3'- TTGGTCTCTATTCTCTGAATATTCC- 5'	58	14
TGLA122	21	5'- CCCTCCTCCAGGTAAATCAGC- 3'		
		3'- AATCACATGGCAAATAAGTACATAC- 5'	58	9
SPS115	15	5'- AAAGTGACACAACAGCTTCTCCAG- 3'		
		3'- AACGAGTGTCCTAGTTTGGCTGTG- 5'	58	13

Table 2

Heterozigosity (Het), Gene Diversity (GD), Polimorphism Informative Content (PIC), Probability of Exclusion (PE), number of alleles and range for pairs base for each microsatellites (MS) on the studied Gyr breed families. Botucatu –SP, 1999.

MS	nº alelos	range (bp)	Het	DG	PIC	PE
TGLA 122*	12	135 - 163	0,5714	0,6046	0,5895	0,4323
TGLA 126*	4	115 - 121	0,4762	0,4809	0,4471	0,3035
ETH 3*	4	113 - 119	0,5119	0,4574	0,4159	0,2769
SPS 115*	5	248 - 256	0,619	0,5238	0,4517	0,3114
ETH 225*	4	139 - 161	0,3452	0,3331	0,3057	0,1878
BM 1824*	4	182 - 188	0,4286	0,4315	0,3963	0,2599
ETH 10*	3	212 - 216	0,5714	0,5696	0,483	0,3403
BMS 2533	7	130 - 146	0,7857	0,7874	0,7574	0,6289
POTCHA	5	135 - 151	0,5119	0,4333	0,4089	0,2705

* microsatellite recommended for bovine paternity tests by ISAG

Table 3

Combined Probability of Exclusion (CPE) in terms of microsatellites number (MS) used, with PE combinations in decreasing order of values. Botucatu –SP, 1999.

MS	1 MS	2 MS	3 MS	4 MS	5 MS	6 MS	7 MS	8 MS	9 MS
BMS2533	0,6289	0,6289	0,6289	0,6289	0,6289	0,6289	0,6289	0,6289	0,6289
TGLA122		0,4323	0,4323	0,4323	0,4323	0,4323	0,4323	0,4323	0,4323
ETH10			0,3403	0,3403	0,3403	0,3403	0,3403	0,3403	0,3403
SPS115				0,3114	0,3114	0,3114	0,3114	0,3114	0,3114
TGLA126					0,3035	0,3035	0,3035	0,3035	0,3035
ETH3						0,2769	0,2769	0,2769	0,2769
POTCHA							0,2705	0,2705	0,2705
BM1824								0,2599	0,2599
ETH225									0,1878
CPE	**0,6289**	**0,7893**	**0,8611**	**0,9043**	**0,9333**	**0,9518**	**0,9648**	**0,9741**	**0,9789**

Table 4

Excluded families in the Paternity Tests and microsatellites markers responsible for the exclusion. Botucatu –SP, 1999.

	TGLA122	TGLA126	BM1824	BMS2533	SPS115	ETH3	ETH10	ETH225	POTCHA
TF / V5 / F5				X				X	
TA / V18 / F18	X			X	X				
TA / V19 / F19	X			X			X		
TA / V20 / F20	X		X	X			X		X
TC / V23 / F23				X				X	
TC / V24 / F24				X				X	
TA / V25 / F25		X	X	X					
TA / V26 / F26				X			X		
TA / V33 / F33	X			X		X	X		X
TA / V34 / F34		X		X				X	
TA / V37 / F37		X		X			X	X	

T = bull , V = cow , F = calf

records is necessary in genetic improvement programs for Gyr breed.

The Table 4 shows the excluded families in the Paternity Tests and microsatellites markers responsible for the exclusion.

Paternity Probability estimation was done in all the families in which paternity exclusion did not occurred (Table 5). Families where exclusion occurred, the Probability of Paternity is null.

Paternity Tests results showed a Probability of Paternity varying between 0.8691 and 0.9999 with 0.9512 on average. Only 8 families reached recommended probability of 0.99.

Table 5
Probability of Paternity (PP) in families in which paternity exclusion did not occurred. Botucatu –SP, 1999.
TA = bull A, TB = bull B, TC = bull C , V = cow , F = calf

TA		TB		TC		TD	
V / F	**PP**	**V / F**	**PP**	**V / F**	**PP**	**V / F**	**PP**
V1 / F1	0,9521	V2 / F2	0,9801	V21 / F21	0,9998	V22 / F22	0,9999
V4 / F4	0,8691	V3 / F3	0,8714	V28 / F28	0,9964		
V9 / F9	0,8691	V6 / F6	0,9228	V29 / F29	0,9972		
V11 / F11	0,9521	V7 / F7	0,9529	V30 / F30	0,9972		
V12 / F12	0,9521	V8 / F8	0,9396	V31 / F31	0,9998		
V14 / F14	0,9521	V10 / F10	0,9574				
V17 / F17	0,8691	V13 / F13	0,8994				
V32 / F32	0,9521	V15 / F15	0,9844				
V35 / F35	0,9521	V16 / F16	0,9905				
V36 / F36	0,9521	V27 / F27	0,9741				
V39 / F39	0,9521	V28 / F28	0,9964				
	V40 / F40	0,8994					
Average	**0,9295**		**0,9474**		**0,9981**		**0,9999**

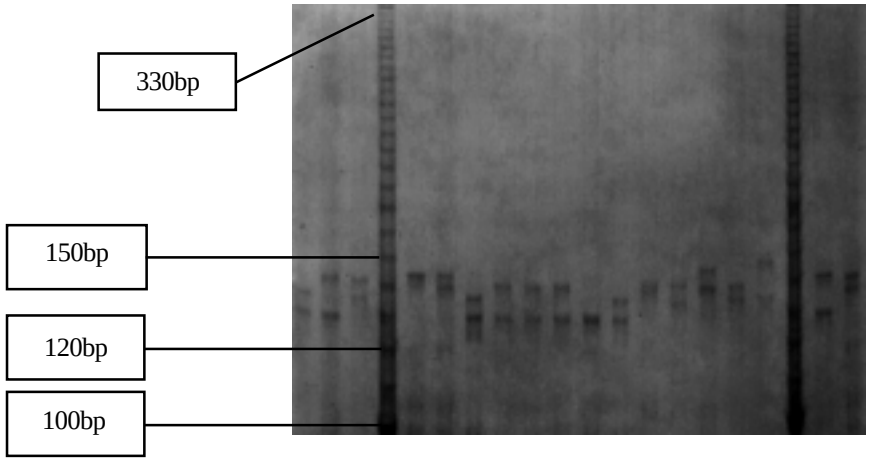

Figure 1
Polyacrylamide denaturing gel electrophoresis of bovine DNA microsatellite revealed with silver staining. Band patterns observed for BMS2533 microsatellite alleles.

Bull A (TA), the more used one in the herd (breeding), had its alleles frequency increased along generations. As a consequence, there was a possible increase in genotypes of bulls compatible to cows genotypes and progeny, decreasing Probability of Paternity of this bull. In bull D (TD) the opposite occurred. So, in confined herds with high levels of endogamic breedings it is necessary to use a greater number of microsatellite markers to reach the optimal Probability of Paternity.

Qui-square tests results for each locus marker was not significant (α=0.05) the genotypic frequencies observed did not differ statiscally from what one would expect, therefore one accept Hardy-Weinberg equilibrium hypothesis for the studied population on Gyr breed.

CONCLUSIONS

PIC, DG, Het and PE results obtained for BMS2533 (not commonly used in Paternity Tests), BM1824 and ETH 225 (recommended for Paternity Tests by ISAG based mainly on studies performed in European cattle) shows that appropriate microsatellites for Paternity Tests in European breeds, maybe are not the most adequate ones for zebu breed and vice versa.

Five or six microsatellites with the same characteristics presented by microsatellite BMS2533 would be enough to perform Paternity Tests on the studied Gyr breed families, lowing costs due to the decrease in material and hand labor necessary. Once the high cost make DNA

Paternity Testing difficult to be performed in commercial scale, this could be the starting point for further studies.

Besides more polymorphic microsatellites for Gyr breed, variants of the PCR technique, as the PCR multiplex, are necessary to decrease costs related to genotyping of the animals and the commercial use of Paternity Tests.

Paternity Testing could be applied in many practical situations such as selections programs which use multiple reproducers on the field performance evaluation programs on young bulls, in families of bulls submitted on a progeny tests and in families of animals registered in the associations, so that we could verify the truthfulness of information given by the producers.

RESUMO

Erros de identificação de paternidade são prejudiciais por reduzir o ganho genético anual e comprometer um programa eficiente de melhoramento genético. O objetivo principal deste trabalho foi avaliar o potencial de uso de nove microssatélites em testes de paternidade e investigar a freqüência de erro de identificação de famílias de um rebanho de animais da raça Gir. No experimento foram utilizadas amostras de sangue de quarenta famílias (touro/ vaca/ bezerro) de animais da raça Gir, Puros de Origem e registrados na Associação Brasileira dos Criadores de Zebu (ABCZ). A maior parte dos microssatélites avaliados neste trabalho são recomendados, para Testes de Paternidade em bovinos, pela Sociedade Internacional de Genética Animal (ISAG). As regiões microssatélites TGLA122, TGLA126, BM1824, BMS2533, SPS115, ETH3, ETH10, ETH225 e POTCHA foram amplificadas por meio da técnica de PCR. Os produtos da amplificação foram separados por eletroforese em gel de poliacrilamida desnaturante. A partir dos dados obtidos foram calculadas as freqüências alélicas, diversidade gênica, conteúdo de polimorfismo informativo e probabilidade de exclusão para cada microssatélite. Também foram calculadas as freqüências genotípicas, heterozigosidade, probabilidade de exclusão combinada e probabilidade de Paternidade nas famílias consideradas. A probabilidade de exclusão combinada para todos os microssatélites estudados foi de 0,9789. Os resultados dos testes de paternidade acusaram erro de identificação em onze das 40 famílias estudadas, ou seja, 27,5% da amostra. A probabilidade de paternidade variou entre 0,8691 e 0,9999, com valor médio de 0,9512.

PALAVRAS-CHAVE: DNA. Microssatélites. Paternidade. Bovinos. Gado Gyr.

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