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Genetic monitoring of a Santa Ines herd using microsatellite markers near or linked to the sheep MHC[1]

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ABSTRACT - This study aimed to analyze genetic diversity in a conservation nucleus of Santa Inês sheep using thirteen microsatellite loci on chromosome 20 (where the Sheep Major Histocompatibility Complex – Ovar-MHC – is found). Seventy three animals from one herd born from 2004 to 2006 were evaluated as a principal nucleus. Seventy one animals from two other herds were used as control comparison. There was a reduction in heterozygosity over the years in relation to the whole population. This may be due to the repeated use of the same sires. The estimates of molecular coancestrality also indicated an increase in genetic similarity between individuals with the herd over the years. A high number of alleles occurred exclusively in the principal nucleus herd, but with a frequency lower than 10%. The Ovar-MHC region of chromosome 20 was shown to be highly polymorphic. Monitoring of the herd over time should be implemented as additional tool for genetic management within the herd.

Key Words: animal genetic resources, genetic diversity, Ovar-MHC, *Ovis aries*, molecular coancestrality, SSR

Monitoramento genético de um rebanho da raça Santa Inês a partir de marcadores microssatélites próximos ou ligados ao MHC ovino

RESUMO - O objetivo neste trabalho foi analisar a diversidade genética de um núcleo de conservação da raça Santa Inês utilizando-se 13 locos de microssatélites localizados no cromossomo 20, onde se encontra o Complexo Maior de Histocompatibilidade ovino – *Ovar-MHC*. Foi avaliado um total de 73 animais nascidos nos anos de 2004, 2005 e 2006 mais 71 animais de outros dois rebanhos como controles. Em geral, constatou-se redução na heterozigosidade dos indivíduos ao longo dos anos em relação à população total, talvez pela baixa rotatividade de reprodutores. As estimativas de co-ancestralidade molecular também evidenciaram aumento da similaridade genética entre os indivíduos do rebanho ao longo dos anos. Há elevado número elevado de alelos privados na população principal, embora esses alelos tenham freqüência menor que 10%. A região do *Ovar-MHC* do cromossomo 20 ovino foi altamente polimórfica e pode ser usada para auxiliar na manutenção de rebanhos. A continuação deste monitoramento ao longo dos anos é desejável e deve ser implantada como ferramenta adicional visando, por exemplo, indicação de acasalamentos e descarte de animais.

Palavras-chave: coancestralidade molecular, diversidade genética, *Ovar-MHC*, *Ovis aries*, recursos genéticos animais, SSR

Introduction

Selection is one of the main tools used in an animal breeding program which identifies animals which should be used to produce the next generation. If this is not carried out with carefully selected criteria, it may, among other things, increase inbreeding within a population. Other factors associated with selection which may affect inbreeding include a structure of populations within and between herds and management directed towards the use of just a few sires (Goyache et al., 2003). This practice reduces the effective size of the population and therefore genetic variability (Kantanen et al., 1999). Additionally, the selection of animals in a random manner may cause fixation of unfavorable alleles in the population (Crisà et al., 2001).

The Major Histocompatibility Complex (MHC) in vertebrates involves a series of highly polymorphic genes which are responsible for the initiation of the immune response of the organism when challenged by pathogens or parasites (Gaudieri et al., 2000; Gruszczynska et al., 2002; Diaz et al., 2005; Geldermann et al., 2006; Schaschl

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et al., 2006; Santucci et al., 2007). Another important trait of this region of the genome is that its structure is relatively conserved between different ruminant species (Ballingall & McKeever, 2005).

According to Klein (1976), the MHC is divided in three regions, class 1 (telomeric), class 2 (centromeric), and class 3 (central). In ruminants there is a division of the Class 2 region in two sub-regions, Class 2a and Class 2b (Andersson et al., 1988). Various studies have shown the existence of polymorphisms in each of these regions (Campbell et al., 1986; Buitkamp et al., 1996; Paterson et al., 1998; Gruszczynska, 1999; Gruszczynska et al., 2000; Kulski et al., 2002). In sheep, the OLA (*Ovine Leukocyte Antigen System*) is localized on chromosome 20 (Scott et al., 1992; De Gotari et al., 1998).

The high variability of the MHC, together with the qualities of microsatellite markers (Powel et al., 1996; Ferreira & Grattapaglia, 1998; Guimarães, 2001), may be an excellent alternative for aiding/monitoring genetic management in herds. In this study the genetic variability of microsatellite loci localized within or close to the MHC region in Santa Inês sheep was studied and their viability tested in analyses of herd management in the Center-West region of Brazil.

Material and Methods

Blood samples from 144 Santa Inês sheep were collected by venal puncture between 2004 and 2006 using 5 mL vacutainers with an anticoagulant (EDTA) and kept refrigerated (4-8ºC) until processing for separation of lymphocytes. The animals were from three different farms:

1) 73 from the University of Brasilia (UnB) farm;

2) 54 from a herd closed for at least 6 years from the Catholic University of Goias (UCG); and

3) 17 from a private herd in the Federal District (DF), Brazil, where there is a constant change of dams and sires from farms different from those above. To evaluate temporal genetic variability of the animals from UnB blood was collected on three different occasions (N=22 in 2004, N=32 in 2005 and N=19 in 2006) and full sibs were excluded (including those collected within the same year). The climate in the region is AW classified by Köppen. Genomic DNA was extracted using the protocol described by Miller et al. (1988). The DNA was then diluted (3 ng/μL), quantified by spectrophotometry and agarose gel 1% and stored until use.

Thirteen microsatellite loci were selected to study the genetic variability within the sheep MHC. These were localized within or in regions adjacent to the sheep MHC (Table 1). Amplification of the microsatellites was carried out using the PCR technique (Polymerase Chain Reaction). 1.5μL of each sample of DNA [3ng/μL] were used; 1.0μL *Tris-HCl* 10% (pH8,4); 0.2μL – 0.5μL $MgCl_2$ [1.0μM – 2.5μM]; 0.8μL *dNTP* [2.5μM]; 1.0μL *BSA* [1.0 mg/mL]; 0.1μL *Taq DNA polymerase* (pHT) [5 Ud/μL]; 0.3 μL *Primer* (F+R) [10 μM]; and water to complete 10 μL final volume. The PCR program included an initial desnaturation step at 94°C for 5 minutes; 35 cycles of desnaturation at 94°C for 1 minute, annealing with temperatures varying between 49°C and 65°C for 45 seconds (depending on the *primer* used), and 72°C for 1 minute for extension. At the end of the 35 cycles a final extension at 72°C for 30 minutes was used to reduce the number of spurious fragments (*stutter bands*).

The PCR amplification success was initially observed in 2% agarose gels coloured with ethidium bromide. Standardized samples with molecular weight of 1kb (Invitrogen) were applied to the gels for comparison of the fragment size obtained. After visualization in agarose, the samples underwent multiplex capillary electrophoresis in an Automatic Sequencer model ABI 3100 or 3700 (Applied Biosystems) to optimize the electrophoresis. The analysis of the electrophoresis and allele genotyping was carried out using the softwares GeneScan and Genotyper (Applied Biosystems). All molecular analyses were carried out in EMBRAPA Genetic Resources and Biotechnology - Cenargen.

Various herd genetic population characteristics were calculated using the softwares Arlequin v.3.1 (Excoffier et al., 2005), Genepop (Raymond & Rousset, 1995), Genalex (Peakall & Smouse, 2005), FSTAT (Goudet 2002) and Molkin (Gutiérrez et al., 2005). These parameters included: mean number of alleles (A_M), allelic richness (A_R), private allelic richness (A_P), as well as observed heterozygosity (Ho), expected heterozygosity (He), Hardy – Weinberg Equilibrium test (EHW) and linkage disequilibrium between each pair of loci in each population studied. Desequilibrium was estimated using permutations carried out between pairs of loci in all herds, and the calculations undergoing the Bonferroni correction in the Genepop program Version 3.1d (Raymond & Rousset, 1995). Fixation indices (or the inbreeding coefficient) within each population (F_{IS}) were also calculated, as well as mean molecular coancestrality in the different populations.

Table 1 - Microsatellite markers and their selected primers on chromosome 20 of Ovis aries with their estimated position on the genetic map

Locus	Synonomyn	Position (v.,4.7)[1]	Primer sequence 5' – 3'	Reference
INRA132	INRA104	9.5	F: aacatttcagctgatggtggc R: ttctgttttgagtggtaagctg	de Gortari et al. (1998)
DYA	DYMS1	11.6	F: aacaccatcaaacagtaagag R: catagtaacagatcttcctaca	Buitkamp et al. (1996)
OarCP73	CP73	21.3	F: aaaactgagaaatattcagatgcaac R: taaacgtccatcaacagaggaaggg	Ede et al. (1995)
OarCP101	CP101	31.2	F: tcataccttaagagagtcgag R:ctatcagacagacagtcagacagtcag	Crawford et al. (1995)
DQBA27	DQB	41.3	F: tccccgcagaggatttcgtg R: tccgccgctgccaggtgaag	Maddox, J.F. Unpublished.
OLADRB	DRB2	46.3	F: ctgccaatgcagagacacaaga R: gtctgtctcctgtcttgtcatc	Schwaiger et al. (1993)
DQA2	OLA-DQA2	55.3	F: cacttatcagctgaccac R: ggtggacacttaccattg	Snibson et al. (1998)
DQA1	OLA-DQA1	55.5	F: accctgactcagctgacc R: catactgttggtagcagc	Snibson et al. (1998)
Bf94_1	Bfms	57.7	F: caacggtctgcaaccgaattacc R: caatccgtgggttggaacacaa	Groth et al. (1995)
BM1818	-	58.9	F: agctgggaatataaccaaagg R: agtgctttcaaggtccatgc	Bishop (1994)
OMHC1	*SMHCC1*	59.6	F: atctggtgggctacagtccatg R: gcaatgctttctaaattctgaggaa	Groth & Wetherall (1994)
TFAP2A	-	71.4	F: ctattaggaggctccaggaacgct R: ctggtcgaacccacggtctctat	Maddox J.F. Unpublished.
OarHH56	*HH56*	73.0	F: gcaacccactcatctctccgtgtc R:aaaacttaagttccagctattaaaatagc	Ede et al. (1994)

[1] Position in centiMorgans on chromosome 20 (http://rubens.its.unimelb.edu.au/~jillm/jill.htm).

Results and Discussion

Analyses of loci DQA2 and TFAP2A were not included in the final results as they did not amplify adequately. Therefore, 11 loci were amplified and analysed. The population of the Catholic University in Goiania had the greatest number of loci out of equilibrium (nine of eleven) while the population from the private farm in the DF (DFC) had only four of the eleven loci outside the expected proportions (Table 2). The BM1818, OarHH56, DQBA27, OarCP101 and DQA1 loci showed deviation from EHW in four of the five populations. Only locus INRA132 did not show significant deviations from EHW. Santucci et al. (2007), studying European sheep breeds with microsatellite markers localized on the MHC and in other regions of the genome, also found significant deviations from EHW. These authors justified that present management practices such as the use of few sires and exchange of sires with neighbouring herds leads to an increase in the complexity for interpretation of genetic structure of populations using traditional measures of population genetics.

These results (significant deviations from expected EHW) are expected as they were studyed in closed herds or herds with small effective population size with low gene flow (ex., UCG), or with directed crossings (UnB and DFC). An alternative explication for this pattern may be due to the stabilizing effect which occurs in this region of the genome (Garrigan & Hedrick, 2003). In this way, some alleles of microsatellite loci may be linked to the genes of the MHC.

Similar to the results for EHW, the population from UCG showed the greatest number of pairs of loci ($P<0.05$ with Bonferroni correction) significantly in linkage disequilibrium, five of the possible 55 combinations or approximately 10%. The other populations, with the exception of the 2006 population from UnB, showed only a single combination in disequilibrium (1.8%). In 75% of the linkage disequilibrium loci combinations the locus OarHH56 was present, which is

Table 2 - Observed (Ho) and expected (He) heterozygosity of eleven loci located on chromosome 20 in five populations of Santa Inês and test for Hardy –Weinberg equilibrium

Locus	DFC[1]		UCG[1]		UnB 2004[1]		UnB 2005[1]		UnB 2006[1]	
	Ho	He	Ho	He	Ho	He	Ho	He	Ho	He
DYA	0,65	0,70	0,58	0,79*	0,50	0,86*	0,60	0,86*	0,63	0,88
BM1818	0,31	0,54	0,40	0,84*	0,39	0,89*	0,29	0,79*	0,22	0,68*
OarHH56	0,47	0,80*	0,64	0,72*	0,55	0,73	0,43	0,67*	0,53	0,74*
DQBA27	0	0,47*	0	0,60*	0	0,44*	0,12	0,60*	0,20	0,19
INRA132	0,38	0,34	0,47	0,52	0,19	0,36	0,45	0,55	0,53	0,56
OarCP101	0,06	0,75*	0,13	0,45*	0,17	0,69*	0,21	0,40	0	0,51*
OMHC1	0,82	0,68	0,63	0,69*	0,68	0,78	0,45	0,79*	0,39	0,59*
DQA1	0,13	0,46*	0,02	0,15*	0,09	0,35*	0	0,12*	0	0,11
OarCP73	0,38	0,52	0,08	0,50*	0,13	0,23	0,21	0,57*	0,06	0,48*
OLADRB	0,73	0,83	0,64	0,79*	0,71	0,84	0,41	0,83*	0,78	0,75
Bf94_1	0,69	0,67	0,08	0,12	0,59	0,628	0,19	0,50*	0,26	0,37*
Mean	0,42	0,61	0,33	0,56	0,36	0,62	0,31	0,61	0,33	0,53*

[1] DFC: private farm in the Distrito Federal; UCG: Universidade Católica de Goiás; UnB: Universidade de Brasília; *significant loci at p<0.01 for deviation from Hardy-Weinberg Equilibrium.

found close to one of the extremities of chromosome 20 of *Ovis aries*. Gametic disequilibrium is the non-random association of different alleles from different loci in the gametes. Linkage disequilibrium may happen due to various genetic factors, from physical linkage (on the same chromossome), due to different selection processes (for example, epistatic selection and genetic lift) or by genetic drift in finite populations. In addition, it may occur due to demographic factors such as co-ancestry, migration and inter-crossing of populations. In the case in question, where the markers are on the same chromosome, physical linkage cannot be discarded. Another process which may influence the results is genetic drift as the population from UCG has a small effective size with probably high rate of inbreeding.

Gametic disequilibrium may also be influenced by the existence of a structure within or between the populations studied. An analysis of molecular variance (AMOVA) was carried out to test the hypothesis of differentiation between the populations. This found that 8.01% (P<0.01) of all observed variation was due to differences between the populations Santa Inês studied. This is a relatively high value for populations within a breed. Paiva (2005) found 11.76% differentiation between 10 sheep breeds in Brazil using 19 microsatellite loci spread over the genome. With this, it is expected that the structure of populations, at least within the Santa Ines breed, influences linkage disequilibrium. Confirmation of this hypothesis would only be possible with a new study with more samples, the least degree of relationship between animals and more markers.

In relation to the observed heterozygosity values it can be seen that the population in UnB from 2005 had the lowest mean value (0.31) and the population from the private farm had the highest observed value (0.42) (Table 2). These values are well below those observed by Paiva (2005), but as they are related to markers on a single chromosome and many of these are probably under selection these values are within expected values. Comparisons with other studies were not carried out as markers used were different.

The analysis of other estimated population parameters (Table 3) showed some patterns which are in agreement with the heterozygosity results. The F_{IS} values show higher inbreeding in the DFC population. On the other hand, the parameters related with variability of alleles are not directly related with heterozygosity, but with molecular coancestry. This is an important result, as it shows that decision making and conclusions based on heterozygosity values are risky and may mask the present genetic state of the population. The population from UnB shows higher mean variability of alleles when compared with the DFC population. The heterozygosity may be influenced by the entrance of new alleles in a population and this is relatively common in commercial properties where sires are exchanged at least once a year.

The final pattern identified was the decrease in genetic variability over the three years in the UnB herd. Practically all the parameters showed the same tendency, but molecular coancestry and presence of private alleles were clearer in showing this tendency (Table 3). Individual mean molecular coancestry values varied from 0.16 (UnB 2005) to 0.64 (UCG) and suggest that it would be impossible to install a program of genetic management to optimize variability of the herds using the reduction in mean individual coancestry. This decrease in genetic diversity and increase in endogamy within the UnB herd can be explained by use of few sires as well as their use with different numbers of females in recent years. In this way, the last year studied may have animals that are more related. Another common practice which may explain this pattern is the permanence of females within the herd born in previous years.

Table 3 - Genetic diversity in Five Santa Ines sheep populations for eleven microsatellite markers on chromosome 20 of Ovis aries. N = number of animals sampled per population, A_M = mean number of alleles, A_R = allelic richness; f_M = mean molecular coancestry; F_{IS}= inbreeding coefficient within each population; A_P <10% = number of specific alleles with frequency lower than 10%; A_P>10% = number of specific alleles with frequency higher than 10%

Population[1]	Indices						
	N	A_M	A_R	f_M	F_{IS}	A_P <10%	A_P >10%
DFC	17	4,45	4,04	0,38	0,27	0	0
UCG	54	5,27	3,93	0,47	0,36	0	0
UnB 2004	22	5,64	4,82	0,36	0,37	2	2
UnB 2005	32	5,64	4,55	0,42	0,46	3	0
UnB 2006	19	5,18	4,21	0,46	0,38	0	0
Média	-	5,24	4,981	0,43	0,38	-	-

[1]DFC, private farm DF; UCG, Universidade Católica de Goiás; and UnB, Universidade de Brasília.

The use of molecular coancestry as indices for monitoring genetic diversity has shown promising and robust results (Royo et al., 2007; Toro et al., 2003; Toro et al., 2002). The results presented here are additional evidence of the use of this parameter as an accessory tool, together with pedigree data for management of animal genetic resources, as well as show that loci close to the MHC may be used as markers of genetic variability. Markers can be used as an aid to selection decision in carrying out directed mating, culling, as well as parentage confirmation. It is important to note that the use of genetic markers for the specific function of maintaining genetic variability would only de desirable, or would have greater response, in herds that never underwent genetic management or that had problems with the collection of production data in some generations. Genetic management using traditional methods based on pedigree are still very efficient for the maintenance of genetic variability within herds (Fernández et al., 2005). As the herd from UnB is experimental in nature, the tendency is to continue to accompany this population for some more years using molecular markers to evaluate their potential and, together with pedigree data, steer matings to increase genetic variability through reduction of coancestry within the herd.

Conclusion

The results show the analytic capacity of this panel of microsatellite markers in capturing genetic variability of the Santa Inês breed. This study also shows how markers can be used to aid in genetic management of sheep herds in Brazil. For breeding programs this panel as well as others may be used to verify the accuracy of pedigrees. The application of these markers would have a greater impact on herds without much production control or those that have problems with pedigree recording.

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