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Application of microsatellite markers for breeding and genetic conservation of herds of Pantaneiro sheep



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ABSTRACT

Background: The aim of the present study was to assess the genetic diversity of Pantaneiro sheep, using microsatellite markers, in order to assist maintenance and management plans, enhance mating systems and reduce the inbreeding rate. A total of 127 animals were genotyped at eight microsatellite loci. They belonged to populations from the Experimental Farm of the Universidade Federal da Grande Dourados (UFGD) (Dourados/MS/Brazil) and Embrapa Pantanal (Corumbá/MS/Brazil).

Results: The population of Pantaneiro sheep from the UFGD exhibited a high mean number of alleles (11.13) and allelic richness (10.66). The polymorphic information content was highly informative in the locus studied, resulting in a mean value of 0.71. Observed heterozygosity was lower than expected for all molecular markers assessed. The analysis of molecular variance showed a differentiation rate of 5.2% between populations.

Conclusions: The results of the statistical parameters indicated that populations of Pantaneiro sheep require special attention on herd management, and it's further necessary to implement breeder exchange programs in order to preserve the genetic variability of these populations. Furthermore, the maintenance of those populations in their typical habitats is rather required to allow different responses from the herds to the interactions between genotype and environment.

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1. Introduction

The genetic group of sheep adapted to the conditions of the Pantanal of the state of Mato Grosso do Sul in Brazil [1] is known as the Pantaneiro sheep. They can be used as an animal genetic resource to improve sheep production for meat and milk in this state. Females of this breed have no reproductive seasonality and are an important role in the performance of their lambs during the period from birth to weaning [2,3]. Lambs have a satisfactory productive potential, in terms of carcass traits and meat quality

[3,4]. In addition, Pantaneiro sheep produce wool, which can be used as feedstock in regional craftwork.

Native Brazilian sheep breeds are characterized by rusticity and adaptability to tropical and subtropical areas within Brazil. Gomes et al. [1] stated that the Pantaneiro sheep exhibit a combination of alleles of wool and woolless sheep breeds from southern and north-eastern regions of Brazil. These animals are phenotypically similar to each other, but differ from other breeds bred in Brazil. Currently, the Pantaneiro sheep is widely diffused in several isolated farms in the state of Mato Grosso do Sul. They live for a long time in Pantanal region and surely faced natural selection mechanism without going through breeding programs. This fact confirms that these sheep are locally adapted [2].

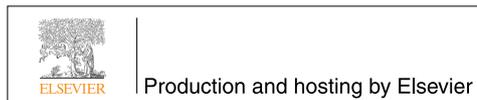
Molecular genetic markers, such as microsatellites, can complement morphological and productive information about genetic resources, contributing to an increase in the efficiency of processes of genetic diversity and genetic purity analysis. In addition, they are able to generate information for the planning of crossings and the selection of genotypes in genetic breeding programs [5]. Microsatellites are suitable for studies of both genetic variability and parentage tests. These markers are co-dominant and frequently have an expected

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heterozygosity above 0.7, which enables discrimination between individuals. Due to the specificity of the PCR assays associated with the high polymorphic content of these markers, it is possible to determine the identity of individuals based on estimates derived from allelic frequencies.

Assessments of genetic diversity using microsatellite markers have become an important tool for conservation programs and those involved in the genetic breeding of animal herds in conservation nuclei. The aim of the present study was to assess microsatellite markers, in terms of characterizing and determining genetic diversity for conservation and management, in order to enhance the mating system and reduce consanguinity in populations of Pantaneiro sheep.

2. Materials and methods

2.1. Animals

Blood samples were collected from 127 animals by jugular venipuncture from each animal using 4.5 mL collection tubes (Vacutainer®) for the genomic DNA extraction, from two conservation nuclei of Pantaneiro Sheep. The conservation nuclei were located on the Experimental Farm of the Universidade Federal da Grande Dourados (UFGD) in the city of Dourados/MS (64 female and 5 male), using a herd formed approximately eight years previously with animals from the ANHANGUERA-UNIDERP Sheep Technological Center in Campo Grande/MS. The other was located at Embrapa Pantanal (47 female and 11 male), using a herd formed approximately 5 years previously, with animals from different places of the Pantanal plain of Corumbá/MS, Brazil.

2.2. Microsatellite loci

Genomic DNA was extracted from blood using 300 μ L of blood which were incubated in microtubules at 60°C with 3 μ L of proteinase K (20 mg = μ L) and 500 μ L of 20% SDS (sodium dodecyl sulfate); chloroform (800 μ L) and a protein precipitation solution (350 μ L) were subsequently added. The microtubules were centrifuged (14,000 rpm) for 10 min and the supernatant transferred to another microtube. One mL of 100% ethanol was added to the pellet and it was centrifuged again, followed by another washing of the precipitate in 70% alcohol. After drying the pellet, 50 μ L of TE buffer (pH 8.7) with RNase (10 ng = μ L) was added. The material was incubated at 37°C for 1 h and stored in a freezer at 20°C. The quantity and purity of the genomic DNA samples was determined using a spectrophotometer (NanoDropND-2000 UV-vis).

PCR reactions were performed for 8 microsatellite loci (CSR247, HSC, OarAE129, MAF214, OarFCB304, OarCP49, SPS113, and D5S2) including those proposed by the Ministry of Agriculture Herds and Provisions of Brazil (MAPA) [6] and markers recommended by the International Society for Animal Genetics (ISAG) [7]. Reactions were performed using a multiplex fluorescent system, with all markers included simultaneously. The PCR was performed in a final volume of 10 μ L, containing: 3.6 μ L of ultrapure water; 1.5 μ L of 10 \times PCR buffer; 1.5 μ L mix of primers; 50 mM MgCl₂; 10 mM dNTPs; 0.4 μ L Platinum® Taq DNA Polymerase (Invitrogen) and 3.0 μ L of template DNA (50–100 ng). Negative controls were used to monitor the reactions. The PCR were realized in thermocycler (Applied Biosystems®), and the thermal profile used was initial denaturing for 7 min at 95°C, followed by 40 cycles of 30 s at 95°C, annealing at 63°C for 90 s and elongation at 72°C for 60 s. A final extension step was performed at 72°C for 30 min. At the end of the amplification, the samples were stored at 4°C. Denatured amplicons were subjected to capillary electrophoresis in a MegaBACE™ 1000 DNA Analysis System (GE Healthcare, USA). Then, a solution with TWEEN and molecular weight marker ET-400 (GE Healthcare) was prepared. Each sample subjected to electrophoresis was composed of 0.3 μ L ROXsize standard, 7.7 μ L TWEEN 20 a 0.1% and

2 μ L of the amplified product. Samples were denatured for 3 min at 94°C and cooled on ice. Sample injection was performed at 3 kV for 80 s and the electrophoresis run was performed at 8 kV for 80 min. Genotyping results for allele discrimination were visualized in the Fragment Profiler program, version 1.2 (GE Healthcare).

2.3. Data analysis

Allele frequency, private alleles and parameters of locus diversity (expected heterozygosity (H_e), observed heterozygosity (H_o), polymorphic information content (PIC), Hardy–Weinberg equilibrium (HWE) and allelic richness (AR)) were estimated for all microsatellites using the CERVUS 3.0 [8], Microsatellite Toolkit, GenAEx [9] and FSTAT [10] software programs.

Estimates for the inbreeding coefficient (F_{IS}) and population structure were assessed by analysis of molecular variance (AMOVA), using the Arlequin program [11].

The pairwise genetic distances between all individuals were estimated by the logarithm proportion of shared alleles (D_{ps}) [12] using the MICROSAT program [13]. The Neighbor joining method (NJ) [14] was used to build a phylogenetic tree based on the genetic distance matrix, with the aid of the PHYLIP computational package [15] and TreeExplorer 2.1.2.

Based on the results of the genotypes of eight microsatellites, the animals were grouped in a given number of populations and probabilistically placed into groups inferred by Bayesian analysis, using the STRUCTURE program [16]. The tests were performed using an admixture model, in which the allelic frequencies were correlated. The programs were set to distinguish samples from two different populations. In order to select the appropriate number of inferred populations, several analyses were conducted with K (number of populations inferred) ranging from 2 to 5, a total of 300,000 interactions (burn-in period of 3000) and three independent replications for each analysis. The real K values were inferred from the magnitude of ΔK and given as a function of K, using the Structure Harvester program [17], according to the model proposed by Evanno et al. [18].

3. Results

Table 1 displays the descriptive statistical analysis of the eight microsatellites for the populations of Pantaneiro sheep studied (127 genotyped animals). All loci exhibited polymorphism resulting in a total number of 100 alleles. The mean number of alleles per locus was 12.5 (ranging from 7 to 21 for the SPS113 and OarCP49 markers, respectively). The loci OarAE129 and SPS113 were in the Hardy–Weinberg equilibrium when populations were analyzed together. However, sheep from Pantaneira Corumbá had shown greater number of markers in equilibrium (D5S2, OarFCB304, OarAE129 e MAF214), when compared to those from Pantaneira UFGD (MAF214 e OarCP49).

The polymorphic information content was highly informative for all loci in the studied populations (overall mean of 0.71). The population of Pantaneiro sheep from the UFGD recorded a higher mean number of alleles per locus (11.13) and private alleles (4.50), as well as greater allelic richness (10.66), genetic diversity (0.73) and observed heterozygosity (0.67) than the population from Embrapa Pantanal. Considering that F_{IS} values are associated with higher homozygosity, the results of the present study indicate that the inbreeding coefficient (F_{IS}) was higher for the Embrapa Pantanal population (0.11) than for the UFGD population (0.09) (Table 2). Among the eight analyzed loci, the population from Embrapa Pantanal showed greater number of loci (4) compared to those from UFGD population (2).

The AMOVA revealed differences (5.2%) between the populations of Pantaneiro sheep studied. The estimates of genetic differentiation based on F_{ST} were significant ($P < 0.002$). The individual dendrogram of both populations was built using the Neighbor joining method, based on

Table 1
Microsatellite genetic variation in populations of Pantaneiro sheep.

	Locus	N	Ho	He	PIC	F _{IS}	HWE	F (null)
Pantaneiro sheep (n = 127)	CDR247	13	0.80	0.80	0.76	-0.01	NS	-0.01
	D5S2	9	0.60	0.60	0.56	0.00	NS	-0.01
	HSC	15	0.77	0.85	0.84	0.10	NS	+0.05
	MAF214	9	0.54	0.58	0.81	0.06	NS	+0.02
	OarAE129	12	0.40	0.69	0.72	0.42	***	+0.26
	OarRCP49	21	0.72	0.78	0.76	0.07	NS	+0.04
	OarFCB304	14	0.60	0.73	0.68	0.18	NS	+0.10
	SPS113	7	0.58	0.65	0.60	0.11	***	+0.07
	Mean (SD)	12.5 ± 4.40	0.62 ± 0.13	0.71 ± 0.09	0.71 ± 0.09	0.12 ± 0.09	2	0.06 ± 0.05

Number of alleles per locus (N), observed heterozygosity (Ho), expected heterozygosity (He), polymorphic information content (PIC), inbreeding coefficient (F_{IS}), Hardy–Weinberg equilibrium (HWE) and F (null) frequency of null alleles. NS – Not significant. *** At equilibrium $P < 0.001$.

the estimated distance as a function of allelic sharing between all animals. Most of the animals were grouped within their population in the dendrogram, although there were a number of exceptions (Fig. 1).

Table 3 shows the proportions of each population attributed to the two groups inferred by the STRUCTURE program, with minimal variance.

The genetic structure of populations was analyzed using Bayesian statistics and the STRUCTURE program, with increasing numbers of populations inferred by the program itself. The $K = 2$ (Fig. 2) corresponds to the K inferred by the Structure Harvester program, according to the methodology proposed by Evanno et al. [18], in which both populations of Pantaneiro sheep were visualized by complex patterns of miscegenation and the similarity between them.

4. Discussion

Data in the present study provides preliminary evidence of the genetic variation of animals belonging to the genetic group of Pantaneiro sheep from two different conservation nuclei in the state of Mato Grosso do Sul, Brazil. The marker analysis indicated that eight loci of microsatellites were considered informative in the analysis of characterization and genetic diversity of Pantaneiro sheep, since they exhibited more than four different alleles per loci [19].

The mean number of alleles per loci was 12.5, which can be considered high when compared with other studies that assessed these markers in ovine breeds [20,21,22]. This finding demonstrates that there is a high variability in the population of Pantaneiro sheep, which can be observed in other locally adapted breeds [23,24], thereby indicating that until now, the population has been exposed to a low artificial selection.

The averages of H_o (0.62) were lower than that of H_e (0.71), which indicates a higher quantity of homozygote and increased heterozygosity, reinforcing the need for studies of genetic management in the herd. The microsatellite markers used for the Pantaneiro sheep were considered highly informative, exhibiting a PIC ranging from 0.56 to 0.84 and a mean value of 0.71 when analyzed together [25]. Arora et al. [26], Santos-Silva et al. [27] and Yilmaz and Karaca [28] obtained PIC values similar to those of the

present study, indicating the genetic efficiency of these markers in studies about Pantaneiro sheep genetic diversity.

Among the studied markers, only two showed to be in HWE (OarAE129 and SPS113) when Pantaneiro sheep populations were analyzed together. The marker OarAE129 showed the largest difference between observed and expected heterozygosity. A significant deviation observed for this marker may be explained by unobserved null alleles leading to high F_{IS} values. Nonetheless, when the same populations were analyzed separately, the Pantaneiro sheep from Embrapa displayed a high number of loci in equilibrium. Probably, this event is related to the different F_{IS} detected in the herds studied, which could be related to differences on the handling of animals, i.e. Pantaneira Corumbá population may have gone through processes of artificial selection and breeding programs distinct of those faced by UFGD herd (Table 1).

The different levels of inbreeding found between the Pantaneiro sheep populations (UFGD and Embrapa) could be related to the mating system (UFGD offers a continuous replace of breeders while in Embrapa it's nonexistent) adopted in different herds. Considering that the reproductive management of these animals is based on phenotypes (herds are constituted of genetically related individuals) and the fact that there is a small number of breeding males, the utilization of molecular tools based on genotypes could enhance the genetics of herds.

The genetic analysis of both Pantaneiro sheep populations demonstrated heterozygosity, an average number of alleles, private alleles, allelic richness, as well as high genetic diversity (Table 2). This is due to the genetic variability found in herds with few management interventions, as was the case in the herd of the present study. Therefore, it is essential to maintain the stock of genetic resources in order to conserve the diversity in locally adapted herds [29,30,31].

Table 2
Genetic diversity of populations of Pantaneiro sheep.

Populations	N	ANA	H _o	H _e	PA	F _{IS}	AR	GD	HWE
UFGD	69	11.13	0.67	0.74	4.50	0.09*	10.66	0.73	2
Embrapa Pantanal	58	8.00	0.57	0.64	1.37	0.11*	7.90	0.64	4

Number of animals (N), average number of alleles (ANA), observed heterozygosity (Ho), expected heterozygosity (He), mean of private alleles (PA), inbreeding coefficient (F_{IS}), allelic richness (AR), genetic diversity (GD), loci number in Hardy–Weinberg equilibrium (HWE).

* $P < 0.003$.

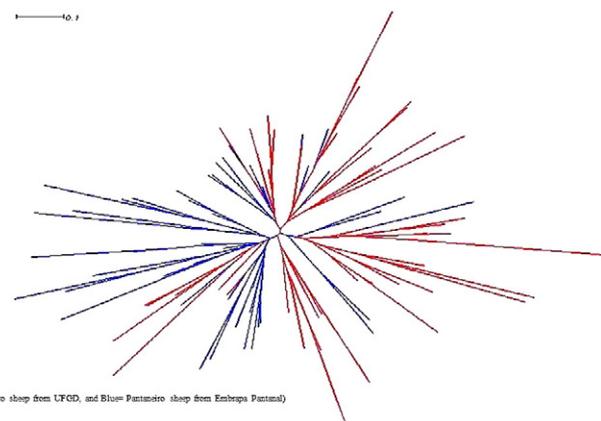


Fig. 1. Individual phylogenetic dendrogram, based on the Neighbor joining method, with the populations of Pantaneiro sheep.

Table 3

Number of individuals (N) per population and proportion of association of each population in each of 5 groups inferred by the program Structure. Associations greater than 0.5 are in bold.

Populations	Inferred clusters		N
	1	2	
UFGD	0.360	0.640	69
Embrapa Pantanal	0.682	0.318	58

Similar data have been reported by Baumung et al. [20] and Tolone et al. [32] when studying naturalized breeds.

Animals from the UFGD population exhibited higher values for genetic diversity, the mean number of alleles and allelic richness than the Embrapa population. This result confirms the formation and management of two conservation nuclei. The UFGD herd was formed from different herds. Mating exchanges from different areas of the Pantanal region are common. Conversely, the Embrapa herd contained a small number of males and was isolated for a long time. Therefore, new males are needed in order to minimize the inbreeding coefficient between populations. Furthermore, the maintenance of those populations in their typical habitats (UFGD – Região de Cerrado/Mata Atlântica e EMBRAPA – Região do Pantanal) is rather required to allow different responses from the herds to the interactions between genotype and environment.

The findings reported above could be associated with the differentiation results of the populations analyzed, where a significant level of genetic variation (i.e. 5.2%) was observed ($P < 0.002$; AMOVA) between Pantaneiro sheep populations. In addition, a recent evolutive process, caused by genetic derivatives and geographical distance, could be included as a cause of this genetic differentiation.

Based on the individual phylogenetic dendrogram from individuals of both populations, it was possible to observe that most of the animals belonging to the same population were grouped in the same cluster, although some animals did not follow this pattern (Fig. 1). Santos-Silva et al. [27] studied 6 Portuguese native breeds (Algarvia, Badana, Galega Bragançana, Galega Mirandesa, Mondgueira and Churra da Terra Quente) and observed that animals from the same population shared the same cluster through the utilization of microsatellites markers. This result could be used to assist management programs for genetic resources aiming to increase genetic variability.

The number of populations, as well as the population structure generated by the STRUCTURE program (Fig. 2), also confirmed that both populations are similar and have a common cluster pattern that shares alleles with few differences. This may be due to genetic derivatives and different management and selection processes. On Table 3 it is possible to observe that in genetically homogeneous populations, tests for individual allocation are more effective. Possibly, better results would be obtained by increasing the number of markers, enabling the use of these tests for the genetic management of populations of Pantaneiro sheep.

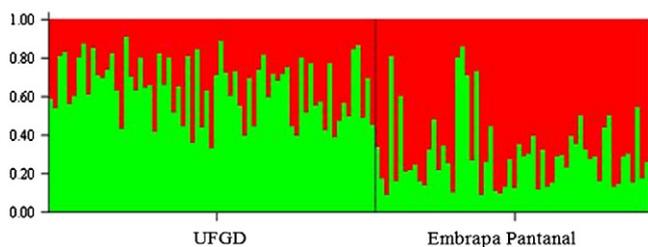


Fig. 2. Individual grouping of 127 Pantaneiro sheep populations from the UFGD and Embrapa, analyzed by the Bayesian statistical method using the STRUCTURE program. Each animal was represented by a vertical line divided into segments classified by size and color, corresponding to the relative proportion of the genome of the animal concerning each group.

In conclusion, the results of the present study indicate that attention should be given to the genetic management of Pantaneiro sheep herds. The population of Embrapa Pantanal has a lower allelic richness and high F_{IS} value indicating a high level of inbreeding. These results are due to the inexistence of a reproductive management and exchange or introduction of new individuals. The UFGD population had lower inbreeding coefficient and superior results of genetic diversity and richness, the management of this herd needs to be taken care of so that these values do not decline over the years. Furthermore, we suggest genotype analyzes including Pantaneiro herds from distinct conservation sites. It is suggested that studies should be conducted in order to implement a genetic management of these valuable genetic resources by inserting in this context exchange between different breeding herds that have different origins. These measures will assist maintenance and improve the genetic base of these animal groups, enabling the conservation of herds with a high genetic diversity in the region.

Conflict of interest

No conflict of interest among the authors.

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Author contribution

Proposed the theoretical frame: ABG, LOS; Conceived and designed the experiments: ABG, FMVJ; Software development: AAE; Contributed reagents/materials/analysis tools: LOS, FMVJ; Wrote the paper: BAC, ABG; Performed the experiments: BAC, ABG; Analyzed the data: AAE, BAC.

References

- [1] Gomes WSG, Araújo AR, Caetano AR, Martins CF, Vargas Junior FM, McManus C, et al. Origem e Diversidade Genética da Ovelha Crioula do Pantanal, Brasil. Simposio de Recursos Genéticos para América Latina y El Caribe. Chapingo, México: Universidad Autónoma Chapingo; 2007.
- [2] Vargas Junior FM, Longo ML, Seno LO, Pinto GS, Barbosa-Ferreira M, De Oliveira DP. Potencial produtivo de um grupo genético de ovinos nativos Sul-mato-grossenses. Pubvet 2011;177:1197.
- [3] Oliveira DP, De Oliveira CAL, Martins EN, De Vargas Junior FM, Seno LO, Pinto GS, et al. Parâmetros genéticos para características de desempenho em ovinos naturalizados Sul-Mato-Grossenses. Semina: Cienc Agrar 2014;35:963–72. <http://dx.doi.org/10.5433/1679-0359.2014v35n2p963>.
- [4] De Lima MC, De Vargas Junior FM, Martins CF, Pinto GS, Nubiato KEZ, Fernandes ARM. Características de carcaça de cordeiros nativos de Mato Grosso do Sul terminados em confinamento. Rev Agrar 2012;5:384–92.
- [5] Faleiro FG. Marcadores genético moleculares aplicados a programas de conservação e uso de recursos genéticos. Planaltina-DF: Embrapa Cerrados; 2007.
- [6] MAPA. Instrução Normativa No. 74 de 20 de Outubro de 2004. Pecuária e Abastecimento, Brasília: Ministério da Agricultura; 2004.
- [7] ISAG. Applied genetics in sheep and goats workshop. 32th international conference on animal genetics. Edinburgh: ISAG; 2010 [Available from Internet: http://www.isag.us/Docs/Applied_GeneticsSheepGoats_CT.pdf]. [cited January, 2014].

- [8] Kalinowski ST, Taper ML, Marshall TC. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Mol Ecol* 2007;16:1099–106. <http://dx.doi.org/10.1111/j.1365-294X.2007.03089.x>.
- [9] Peakall R, Smouse PE. GenALEX 6.5: Genetic analysis in Excel. Population genetic software for teaching and research—an update. *Bioinformatics* 2012;28:2537–9. <http://dx.doi.org/10.1093/bioinformatics/bts460>.
- [10] Goudet J. FSTAT (Version 1.2): A computer program to calculate f-statistics. *J Hered* 1995;86:485–6.
- [11] Schneider S, Roessli D, Excoffier L. Arlequin version 2000: A software for population genetics data analysis. Geneva, Switzerland: Genetics and Biometry Laboratory, University of Geneva; 2000.
- [12] Bowcock AM, Ruiz-Linares A, Tomfohrde J, Minch E, Kidd JR, Cavalli-Sforza LL. High resolution of human evolutionary trees with polymorphic microsatellites. *Nature* 1994;368:455–7. <http://dx.doi.org/10.1038/368455a0>.
- [13] Minch E, Ruiz-Linares A, Goldstein DB, Feldman MW, Cavalli-Sforza LL. Microsat2: A computer program for calculating various statistics on microsatellite allele data. Stanford, CA: Department of Genetics, Stanford University; 1998.
- [14] Saitou N, Nei M. The Neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987;4:406–25.
- [15] Felsenstein J. PHYLIP: Phylogeny inference package. Seattle, WA: University of Washington; 2002.
- [16] Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. *Genetics* 2000;155:945–59.
- [17] Earl DA, Von Holdt BM. STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv Genet Resour* 2012;4:359–61. <http://dx.doi.org/10.1007/s12686-011-9548-7>.
- [18] Evanno G, Regnaut S, Goudet J. Detecting the number of clusters of individuals using the software structure: A simulation study. *Mol Ecol* 2005;14:2611–20. <http://dx.doi.org/10.1111/j.1365-294X.2005.02553.x>.
- [19] Barker JSF. A global protocol for determining genetic distances among domestic livestock breeds. Proceedings of the 5th world congress on genetics applied to livestock production, 21. Ontario, Canada: Guelph; 1994. p. 501–8.
- [20] Baumung R, Cubric-Curik V, Schwend K, Achmann R, Solkner J. Genetic characterization and breed assignment in Austrian sheep breeds using microsatellite marker information. *J Anim Breed Genet* 2006;123:265–71. <http://dx.doi.org/10.1111/j.1439-0388.2006.00583.x>.
- [21] D'Angelo F, Albenzio M, Sevi A, Ciampolini R, Cecchi F, Ciani E, et al. Genetic variability of the Gentile di Puglia sheep breed based on microsatellite polymorphism. *J Anim Sci* 2009;87:1205–9. <http://dx.doi.org/10.2527/jas.2008-1224>.
- [22] Calvo JH, Alvarez-Rodriguez J, Marcos-Carcavilla A, Serrano M, Sanz A. Genetic diversity in the *Churra tensina* and *Churra lebrijana* endangered Spanish sheep breeds and relationship with other Churra group breeds and Spanish mouflon. *Small Rumin Res* 2011;95:34–9. <http://dx.doi.org/10.1016/j.smallrumres.2010.09.003>.
- [23] Paiva SR, Mariante AS, Blackburn HD. Combining US and Brazilian Microsatellite data for a meta-analysis of sheep (*Ovis aries*) breed diversity: Facilitating the FAO global plan of action for conserving animal genetic resources. *J Hered* 2011;102:697–704. <http://dx.doi.org/10.1093/jhered/esr101>.
- [24] Souza CA, Paiva SR, McManus CM, Azevedo HC, Mariante AS, Grattapaglia D. Genetic diversity and assessment of 23 microsatellite markers for parentage testing of Santa Inês hair sheep in Brazil. *Genet Mol Res* 2012;11:1217–29. <http://dx.doi.org/10.4238/2012.May.8.4>.
- [25] Botstein D, White RL, Skolnick M, Davis RW. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am J Hum Genet* 1980;32:314–31.
- [26] Arora R, Bhatia S, Mishra BP, Joshi BK. Population structure in Indian sheep ascertained using microsatellite information. *Anim Genet* 2011;42:242–50. <http://dx.doi.org/10.1111/j.1365-2052.2010.02147.x>.
- [27] Santos-Silva F, Ivo RS, Sousa MCO, Carolino MI, Ginja C, Gama LT. Assessing genetic diversity and differentiation in Portuguese coarse-wool sheep breeds with microsatellite markers. *Small Rumin Res* 2008;78:32–40. <http://dx.doi.org/10.1016/j.smallrumres.2008.04.006>.
- [28] Yilmaz O, Karaca O. Karya Koyunlarda Mikrosatellit İřaretleyicilerle Babalık Testi. *Kafkas Univ Vet Fak Derg* 2012;18:807–13.
- [29] Mariante AS, Egito AA, Albuquerque MSM, Paiva SR, Ramos AF. Managing genetic diversity and society needs. *Rev Bras Zootec* 2008;37:127–36. <http://dx.doi.org/10.1590/S1516-35982008001300016>.
- [30] Dalvit C, De Marchi M, Zanetti E, Cassandro M. Genetic variation and population structure of Italian native sheep breeds undergoing in situ conservation. *J Anim Sci* 2009;87:3837–44. <http://dx.doi.org/10.2527/jas.2008-1682>.
- [31] McClean L, Waterman L, Roberts C. Genetic analysis of three populations of barbados blackbelly sheep at microsatellite loci. *J Agric Sci Technol* 2011;1:1187–91.
- [32] Tolone M, Mastrangelo S, Rosa AJM, Portolano B. Genetic diversity and population structure of Sicilian sheep breeds using microsatellite markers. *Small Rumin Res* 2012;102:18–25. <http://dx.doi.org/10.1016/j.smallrumres.2011.09.010>.