

## Molecular characterization of parasite resistant/susceptible Uruguayan Merino lambs

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**ABSTRACT.** Gastrointestinal parasites (GIP) are one of the main sanitary and economic limitations for Uruguayan sheep production. Many authors suggest that there would be a possible relationship between microsatellites and sheep parasite resistance. Based on extreme fecal egg worm count expected progeny difference, 50 resistant (R) and 50 susceptible (S) lambs were chosen from a Merino flock. In order to investigate the genetic variability and structure in this flock, four polymorphic microsatellites (McM214, McM130, McM357 and CSRD2138) which are potentially associated with QTL for parasite resistance were analyzed. The number of alleles varied from 8 to 13 showing different frequencies, polymorphic information content values were >0.5 and the observed heterozygosity ranged from 0.735 to 0.773. A considerable genic and genotypic differentiation between R and S subpopulations was observed. McM214 showed a significant deviation from Hardy-Weinberg equilibrium from whole and S population. Fixation Indices ( $F_{IS}$ ) values indicated also a heterozygote excess in the entire population. McM214 showed linkage disequilibrium with McM357 in the R sample, and with McM130 in the S sample. Population structure analysis proved the origin of two clusters (subpopulations) from two different lines ( $K=2$ , similarity coefficient =0.979). Polymorphism of these markers could be used in association analysis with GIP resistance / susceptibility.

**Key words:** disease resistance, microsatellite, linkage disequilibrium, population genetics, animal breeding, sheep.

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## Caracterización molecular de corderos Merino uruguayo resistentes/ susceptibles a parásitos.

**RESUMEN.** Parasitosis gastrointestinales (PGI) causadas por nematodos constituyen una de las principales limitantes sanitario-económicas para la producción ovina Uruguaya. Muchos autores sugieren una posible relación entre la resistencia a parásitos y los microsatélites. De una población de merino de 357 individuos, se escogieron 50 animales resistentes (R) y 50 animales susceptibles (S) en base a la diferencia esperada en la progenie para conteo de huevos por gramo de materia fecal. Cuatro microsatélites (STR), McM214, McM130, McM357 y CSRD2138, potencialmente asociados a QTL a resistencia a parásitos. La frecuencia y el número de alelos varió para cada marcador desde 8 hasta 13, índices de contenido polimórficos todos mayores a 0.5, la  $H_o$  presentó valores entre 0.735-0.773. Evidenciando el elevado polimorfismo de estos microsatélites. Se observó diferenciación génica y genotípica significativa entre las dos subpoblaciones R y S. McM214 presentó un desvío significativo del equilibrio Hardy-Weinberg. Los valores de los índices  $F_{IS}$  indicaron en general un aumento de heterocigotos en toda la población. McM214 presentó ligamiento con el McM357 en la muestra R y con el McM130 en la muestra S. El análisis de la estructura poblacional evidenció el origen de las dos subpoblaciones a partir de dos líneas diferentes ( $K=2$ , coeficiente de similitud (SC)=0.979). La variabilidad genética detectada en esta población para los microsatélites analizados permitiría utilizarlos en análisis de asociación con resistencia/susceptibilidad a PGI. Este trabajo ofrece perspectivas interesantes para la incorporación del uso de marcadores moleculares en el mejoramiento ovino en el Uruguay.

**Palabras clave:** resistencia a enfermedades, microsatélites, desequilibrio de ligamiento, genética, de poblaciones, mejora genética, ovinos.

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## Introduction

In Uruguay, gastrointestinal parasites (GIP) represent one of the main sanitary and economic restrictions for sheep production in pasture based systems (Castells *et al.*, 1995). The economic impact of GIP is given by the prevention costs (i.e. antihelminthic), and production losses due to animals morbidity and mortality (Sykes, 1994). Furthermore, the increasing development of antihelminthic resistance, has determined the search for complementary control methods and its incorporation into an Integrated Parasites Control (Castells *et al.*, 2002). An option for stud breeders is the selection of resistant animals to GIP by the evaluation of the Expected Progeny Differences (EPD) for Faecal Worm Egg Count (FEC). Some countries have included the genetic evaluation of FEC (e.g. WormFEC in New Zealand, Nemesis in Australia). In fact, this is already being done in some studs of Uruguayan Merino breed (Ciappesoni, *et al.* 2010). Because FEC is a laborious procedure and infected

animals are required, an alternative solution would be molecular markers assisted selection (MAS) for parasite resistance in breeding programs. Microsatellites (STRs) have been one of the main described markers which might be associated with quantitative trait *loci* (QTL) for resistance/susceptibility to parasites in sheep. Actually, many different chromosomal regions like OAR1, OAR3, OAR5, OAR6, OAR11 and OAR12, have been reported to show significant association with those QTLs (Hulme *et al.*, 1993, Beh *et al.*, 2002, Benavides *et al.*, 2002). Including MAS in breeding programs, requires a previous study of genetic variability and association in the population. The purpose of this study is to analyze the polymorphism of four STRs: McM130, McM214, McM357 and CSRD2138, to study the genetic variability and structure of a Merino population, in order to provide data for future breeding programs and improve the genetic resistance to GIP.

## Materials and methods

### *Genetic and phenotypic evaluation*

A population of 350 Uruguayan Merino lambs (180 males and 170 females), genetically evaluated for resistance to GIP (FEC EPD), from the Selection Nucleus "Glencoe" (NFG) (INIA Tacuarembó, Uruguay) were studied. Two FEC measures were used for the evaluation, (at 7.5 and 9.5 months of age in average) corresponding to independent parasite cycles, with a Naftalophos (Baymetin®) anthelmintic treatment between both infections. After weaning, FEC values of all animals were reduced to zero after drenching. Fortnightly FEC records were taken from 20 lambs randomly selected. When FEC values exceed 400-500, the whole population was sampled obtaining the FEC1 record. The procedure was repeated after a second anthelmintic treatment to have the FEC2. FEC was determined using the modified McMaster technique (Whitlock, 1948) with a sensibility of 100. The genetic evaluation was based on a repeatability model described by Ciappesoni *et al.* (2010).

### *Criteria of animal selection*

One hundred Merino lambs from eight families of paternal half-sib, were studied from the NFG. Selective genotyping (Lander and Botstein, 1989) was utilized to select 50 animals considered as resistant (R) and 50 as susceptible (S), with the lowest and highest FEC EPD values, respectively.

### *Microsatellite analysis*

DNA extractions were made with DNAzol® (Invitrogen) from peripheral blood with EDTA K<sub>3</sub>. Multiplex PCR amplifications (Table 1) were performed in a Corbett Research (model CG1-T) by the following program: a initialization step of, 10 min at 94°C, then 35 cycles of 30 s at 94°C, 61 s at 61°C and 1 min at 72°C, and a final extension of 20 min at 72°C. The genotyping was done in an ABI-PRISM™ 310 (Applied Biosystems, Inc. Foster City, CA-USA) and allelic variation was analyzed with the Genotyper software v. 3.7 (ABI PRISM).

### *Statistical analysis*

Genetic variability and constitution for each *locus* pair, within each subpopulation (R and S) and in the whole population (R+S) were analyzed. The allele and genotype frequencies, and the genotypic (Goudet *et al.*, 1996) and genic (Rousset and Raymond, 1995) differentiation were also calculated for both subpopulations, and p-values were obtained computing Fisher's exact test. Hardy-Weinberg equilibrium was evaluated by the probability test (Haldane, 1954; Guo and Thompson, 1992; Weir, 1996) and the U test was used to calculate for Heterozygote excess and deficiency (Rousset and Raymond, 1995). Expected numbers of heterozygotes was computed using Levene's correction (Levene, 1949). In addition, Fixation Indices ( $F_{is}$ ) values for each allele and global estimate over alleles, were

Table 1. Nomenclature, position, primer sequences and references of the used microsatellites.

STR	Chromosome	Primer sequences (5'-3')	References
McM214	6	AAGCGACTCAGGAGCAGCAGAATGCTTGCATTTA TCAAAAAGCC	Hulmeet <i>et al.</i> 1995
McM357	1	ATCTCTTTIGCTCACCAATTAAGCA CGAAAACAT TGAGTGTGCG	Hulmeet <i>et al.</i> 1994
McM130	1	AAACTTTGTGCTIGTTGGGTATCCTCACCTCTGCCTT TCTATCTCTCT	Hulmeet <i>et al.</i> 1994
CSR2138	5	AGATGCTATTCCAAACACAGTCCCCATGGGGT CACAAAGAGTTGGACA	Drinkwater <i>et al.</i> 1997

computed following Weir and Cockerham (1984). Linkage disequilibrium (LD) test (Weir, 1996) for each *locus* pair within each subpopulation and a global test (Fisher's method) for each pair of *loci* across subpopulations, were performed. All mentioned parameters were estimated with the *GENEPOP* software version 4.0.7 (Rousset, 2007), using a Markov chain algorithm implemented with 100000 dememorizations, 10000 batches and 5000 iterations per batch. Polymorphic information content (PIC) was

calculated according to Botstein *et al.* (1980). The *Structure* software version 2.3.3 (Pritchard *et al.*, 2000) was used in order to evaluate levels of subdivision between the two subpopulations. The range of possible clusters ( $K$ ) tested was from one to eight, and 10 runs were done for each  $K$ . The similarity coefficient (SC), like a measure of the constancy over runs, was used to define the population structure. This coefficient was obtained with SIMCO package for R software (Jones, 2007).

## Results

Table 2 details the number of lambs per sire classified as resistant or susceptible (according to FEC EPD) and presents the descriptive statistics for FEC EPD by subpopulation. The sires with more offspring were P8 and P5 with 29 and 31 lambs, respectively. In addition, P5 is the sire of the rams P1 and P3, and P8 is the sire of P2. In the last National Genetic Evaluation of Merino Breed in Uruguay (Gimeno and Ciappesoni, 2010), the sire of the studied animals that presented the highest FEC EPD was P5 (+0.248 LnFEC) and the lowest was P8 (-0.043 LnFEC).

Some of the alleles presented in the population (Figure 1), were exclusively provided from the maternal line like: I, K, O and U of McM214, L, M, N, Q and R, of McM357, L, M, P, R and U of McM130 and I, J, K, O and P of CSR2138. The studied STRs presented high variability, showing from eight to 13 alleles (Figure 1). The high degree of polymorphism was also demonstrated by the allele frequencies, the PICs values (all >0.50) and the observed Heterozygosity ( $H_o$ ) (all >0.735) for the total population. Some alleles had higher frequencies in one of the subpopulation than in the other. For instance, McM214\*U and McM130\*K were 10 and six times more frequent in R than S subpopulation, respectively. Particularly the frequency of McM214\*J was about 3 times higher in the R sample (Figure 1). However, McM130\*H and CSR2138\*K, showed similar frequencies in both

subsets. Other alleles were detected in only one subpopulation with very low frequencies (<0.04) (Figure 1). The genic and genotypic differentiation tests (Table 3) between R and S subpopulations, showed significant differences ( $p < 0.05$ ) for all STRs. The *locus* McM214 showed a significant excess of heterozygotes ( $p < 0.01$ ) in the S sample and in the total population (Table 4), corresponding to the value and sign of the estimated  $F_{IS}$  (-0.024) (Table 5). According to the classification of Hartl and Clark (1997), the  $F_{IS}$  values (Table 5) indicate generally low to moderate inbreeding. The greatest  $F_{IS}$  value is for the McM214 in the S subpopulation, indicating increased inbreeding. In this subpopulation the ram P5 is the sire or grandsire of most of the lambs. In addition, almost of the  $F_{IS}$  values were negative (excess in heterozygotes), with the exception of *loci* McM130 (S sample), CSR2138 (R sample) and McM357 (R sample and total population) that presented a positive value (reduction in heterozygotes).

As is presented in Table 6, microsatellites mapped on the same chromosome (McM130 and McM357) showed no significant LD in both samples ( $p > 0.05$ ). However, a significant LD ( $p < 0.05$ ) was showed between McM214 and McM357 in the R sample and with McM130 in the S sample; and between McM130 and CSR2138 in the S sample. In addition, significant LD between McM214 and McM357

Table 2. Number of lambs per ram and descriptive statistics for FEC EPD by subpopulation.

Subpopulation	Number of lambs per sire									FEC EPD (LnFEC)			
	P1	P2	P3	P4	P5	P6	P7	P8	Total	Mean	S.D.	Min.	Max.
Resistant	1	7	4	0	2	0	8	28	50	-.130	.01	-.30	-.047
Susceptible	0	2	12	2	29	1	3	1	50	.201	.041	.139	.328

Note: S.D. Standard deviation, Min. Minimum, Max. Maximum.

( $p=0.0474$ ), McM130 and McM214 ( $p=0.0120$ ), and between McM130 and CSRD2138 ( $p=0.0444$ ), was observed when the whole population was evaluated.

In population structure analysis, SCs ranged from 0.365 to 0.979. The highest value of SC corresponded to  $K = 2$ , therefore it was considered the most appropriate number of subpopulations. In this case,

61% of resistant individuals were included in cluster one, and 66% of susceptibles in cluster two (Figure 2). Furthermore, when three hipotetic subpopulations ( $K=3$ ,  $SC=0.956$ ) were analyzed, resistant animals continued tightly clustered in cluster one, while susceptible lambs showed a similar probability to belong to cluster two or three (Figure 2).

## Discussion

All STRs were highly polymorphic, and showed significant differences in the genotypic and allele frequencies between R and S subpopulations. These differences could be explained by the existence of alleles or associated haplotypes with resistance or

susceptibility to GIP in each subpopulation, that is reflected in FEC EPD. The different allelic frequencies observed in Figure 1 were supported by genic and genotypic differentiation test (Table 3). Another factor that could be conditioning this result is the sire

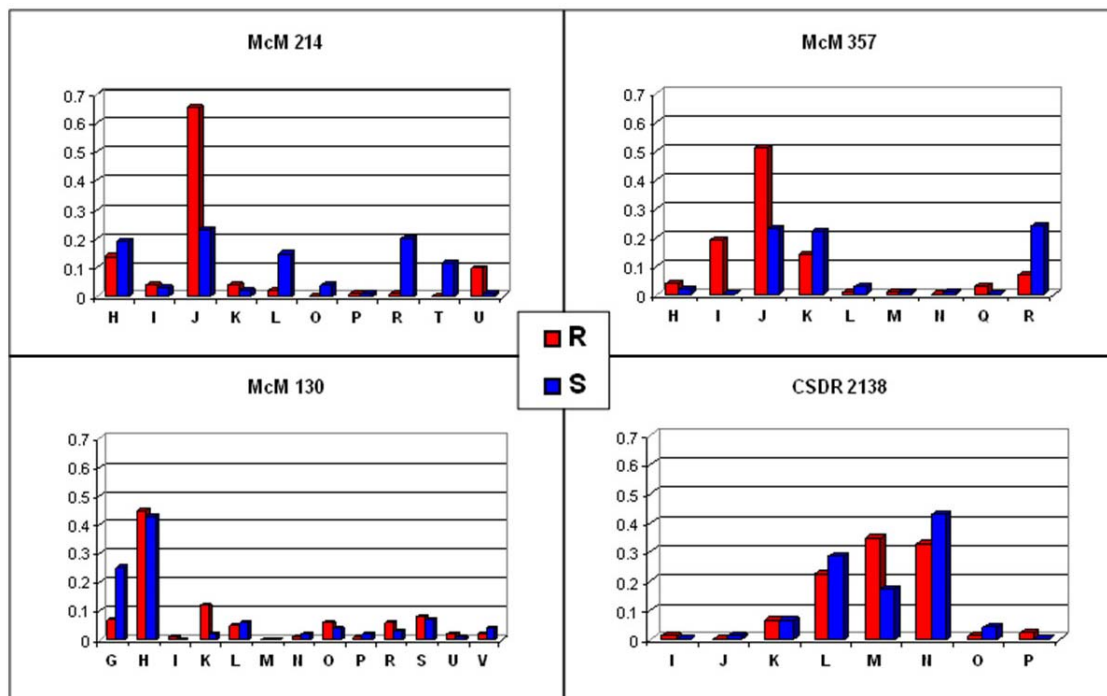


Figure 1. Allele frequencies distribution between resistant (R) and susceptible (S) subpopulations for McM214, McM357, McM130 and CSRD2138 STRs.

Table 3. Genic and genotypic differentiation analysis for each microsatellite.

STR	Genic differentiation		Genotypic differentiation	
	p-value	S.E.	p-value	S.E.
McM214	<.0001	<.00001	<.0001	<.00001
McM357	.0002	.00001	.0003	.00001
McM130	.0315	.00015	.0259	.00013
CSRD2138	.0392	.00018	.0353	.00013

Note: S.E. Standard Error

Table 4. Hardy-Weinberg test for each STR between and within subpopulations.

STR	Statistic test	Population					
		Resistant		Susceptible		Total	
		p-value	S.E.	p-value	S.E.	p-value	S.E.
McM214	Def. of Heterozygotes	.8742	.0011	.9986	.0001	.9990	<.0001
	Ex. of Heterozygotes	.1767	.0012	.0021	.0001	.0009	<.0001
	Probability test	.1084	.0010	.1593	.0008	.0874 <sup>1</sup>	
McM357	Def. of Heterozygotes	.1628	.0007	.5208	.0012	.2659	.0010
	Ex. of Heterozygotes	.8369	.0007	.4811	.0012	.7340	.0010
	Probability test	.0303	.0003	.5177	.0009	.0801 <sup>1</sup>	
McM130	Def. of Heterozygotes	.9777	.0004	.5716	.0020	.9175	.0008
	Ex. of Heterozygotes	.0523	.0007	.4520	.0019	.0819	.0009
	Probability test	.7293	.0016	.6295	.0018	.8144 <sup>1</sup>	
CSRD2138	Def. of Heterozygotes	.4450	.0012	.7278	.0007	.6434	.0009
	Ex. of Heterozygotes	.5657	.0012	.4520	.0019	.3550	.0009
	Probability test	.8787	.0004	.2841	.0006	.5967 <sup>1</sup>	

Note: S.E. Standard Error. <sup>1</sup>Fisher method computed

Table 5. Observed and expected heterozygosities (Ho, He), Polymorphic Information Content (PIC) and Fixation Indices (F<sub>S</sub>) for each STR by subpopulation (Resistant =R, Susceptible =S and Total).

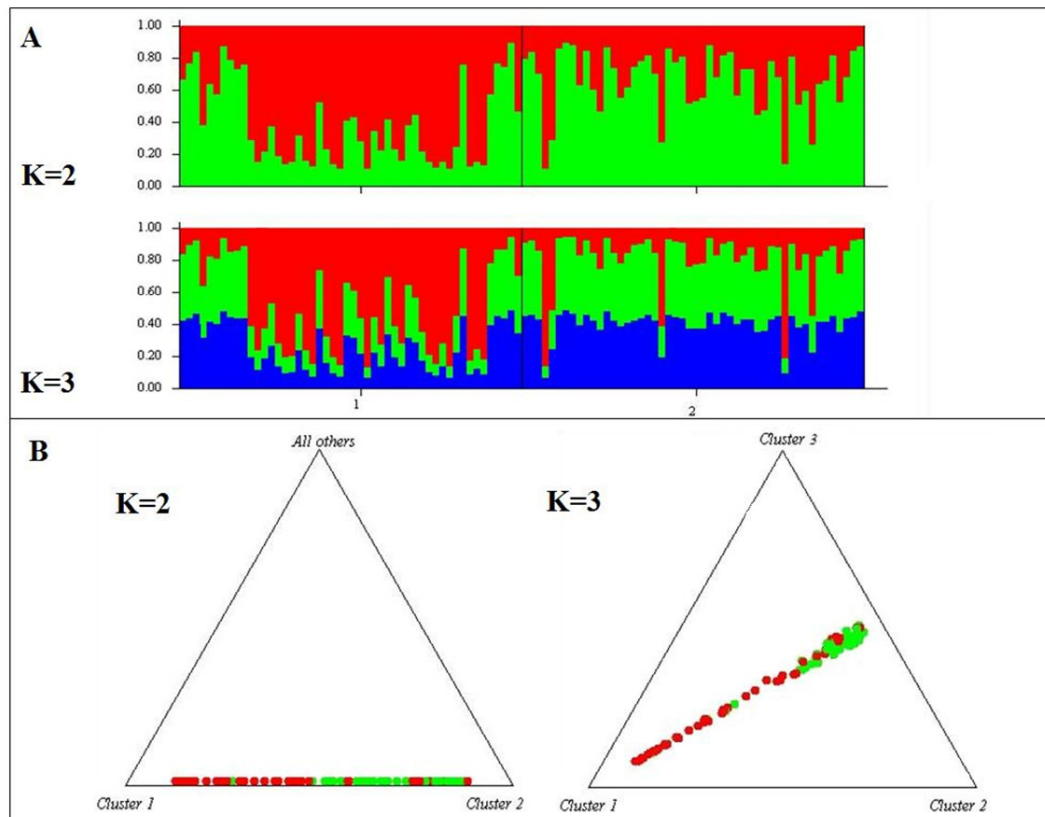
STR	McM214			McM357			McM130			CSRD2138		
	R	S	Total	R	S	Total	R	S	Total	R	S	Total
Ho	.580	.960	.770	.680	.796	.737	.830	.720	.773	.714	.755	.735
He	.553	.839	.752	.683	.791	.762	.757	.746	.759	.726	.706	.724
PIC	.522	.809	.723	.641	.747	.723	.731	.708	.735	.667	.650	.671
F <sub>S</sub>	-.049	-.145	-.024	.005	-.006	.033	-.096	.035	-.019	.015	-.070	-.015



**Table 6.** Test of significance for genotypic linkage disequilibrium for Resistant and Susceptible subpopulations.

Subpopulation	Locus #1	Locus #2	p-value	S.E.
Resistant	McM214	McM357	.0162	.0006
	McM214	McM130	.4279	.0027
	McM357	McM130	.1474	.0020
	McM214	CSRD2138	.3786	.0021
	McM357	CSRD2138	.3220	.0023
	McM130	McM138	.2421	.0021
Susceptible	McM214	McM357	.5034	.0028
	McM214	McM130	.0038	.0003
	McM357	McM130	.3752	.0029
	McM214	CSRD2138	.5967	.0021
	McM357	CSRD2138	.2807	.0018
	McM130	CSRD2138	.0312	.0008

Note: S.E. Standard Error



**Figure 2.** Population structure analysis estimated for the four STRs with two and three cluster. A) Each individual is represented by a vertical line, which is divided by K colors segments representing the estimated fraction of belonging to each cluster. The black line separates individuals from different subpopulations, identified by 1 = Resistant and 2 = Susceptible. B) Each triangle vertices relates to different cluster, dots represent individuals from each subpopulation: green (susceptible) and red (resistant).

effect. Alleles present in the S subpopulation predominantly come from the ram P5, and in the case of the R sample came from the ram P8.

Although, this population is under strong selection pressure, that is primarily focused on reducing fiber diameter, with not relevance in FEC EPD, the low levels of endogamy are probably because mating are diagrammed to avoid inbreeding, as detailed in Ciappesoni *et al.* (2009). Additionally, the genetic correlation between fiber diameter and FEC estimated in the Uruguayan Merino is unfavorable but of low magnitude ( $-0.177 \pm 0.026$ , Ciappesoni *et al.*, 2010). Significant LD between STRs mapped in different chromosomes, could be explained by the existence of LD within the families tested or at a population level. Probably the allele frequencies in these *loci* could be affected by the action of natural (Bodmer and Parsons, 1960) and/or artificial selection, having advantage those haplotypes associated with QTL for resistance to GIP. The no significant LD between *loci* located at the chromosome one (i.e. McM357 and McM130), was probably due to the distance between them (87cM, Maddox *et al.*, 2001). When these *loci* are situated on different chromosomes, these associations can be spurious and disappear in future generations. Probably, the LD found is caused by the effect of inbreeding within families and the population structure (sire effect).

Population structure analysis revealed the origin of two subpopulations from two different lines ( $K=2$ ). These results are consistent because, animals of cluster one are mostly sons or grandsons of P8 ram (resistant) and the rest of lambs (cluster two) are

mainly sons or grandsons of the P5 ram (susceptible); defining the population structure by origin. Nevertheless, not all lambs from a sire were always resistant or susceptible. This population can not be subdivided into a greater number of clusters, because of the similar alleles frequencies in both samples (R and S), or because could be more associated markers to this quantitative trait. Therefore, more markers would be required to a better definition of the population structure.

In conclusion, the results obtained in this work show the variability of the selected markers and differences in the distribution of alleles in samples selected by FEC EPD. This indicates that STRs would be a useful tool for association studies, in this case for resistance/susceptibility to GIP, and to evaluate the potential effect on FEC. However, further studies using different statistics models are needed to evaluate the STRs allele substitution effect, that would confirm that these polymorphisms are responsible for the differences observed in FEC EPD. For example, it has been proved that STRs located in the interleukins 3, 4 and 5 regions of OAR5, have indicated that an allele of the CSRD 2138 was associated with a FEC reduction of 22% (Benavides *et al.*, 2002).

The LD observed in the presented study should be confirmed in other populations, as well as other generations of the NFG (different families) or commercial flocks. Finally, these results provide interesting perspectives for the incorporation of molecular markers as an additional tool in sheep breeding in Uruguay, specifically in relation to GIP resistance.

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