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 ($F_{st}$) 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.

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 feca. 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 Ho presentó valores entre 0.735-0.773. Evidenciando el elevado polimorfismo de estos microsatélites. Se observó diferenciación génica y genotípica significativa entre las dos subpopulaciones R y S. McM214 presentó un desvió significativo del equilibrio Hardy-Weinberg. Los valores de los índices $F_{st}$ 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 subpopulaciones 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.
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. anthelmintic), and production losses due to animals morbidity and mortality (Sykes, 1994). Furthermore, the increasing development of anthelmintic 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 K3. 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 Bisystems, 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 (FIs) values for each allele and global estimate over alleles, were
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 CSRD2138. 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 (Ho) (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 CRDS2138*K, showed similar frequencies in both subsets. Other alleles were detected in only one subpopulation with very low frequencies (<0.04) (Figure1). 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<sub>IS</sub> (-0.024) (Table 5). According to the classification of Hartl and Clark (1997), the F<sub>IS</sub> values (Table 5) indicate generally low to moderate inbreeding. The greatest F<sub>IS</sub> 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<sub>IS</sub> values were negative (excess in heterozygotes), with the exception of loci McM130 (S sample), CRDS2138 (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 CRDS2138 in the S sample. In addition, significant LD between McM214 and McM357

<table>
<thead>
<tr>
<th>STR</th>
<th>Chromosome</th>
<th>Primer sequences (5´-3´)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>McM214</td>
<td>6</td>
<td>AAGCGACTCAGGACAGACAGATGCTGCAATT</td>
<td>Hulme et al. 1995</td>
</tr>
<tr>
<td>McM357</td>
<td>1</td>
<td>ATCTCTTGTGCTGATTTAACAACTAAGCA</td>
<td>Hulme et al. 1994</td>
</tr>
<tr>
<td>McM130</td>
<td>1</td>
<td>AAATTGCTGTTGGGTGTAATCTGACCTGCTCTTC</td>
<td>Hulme et al. 1994</td>
</tr>
<tr>
<td>CSRD2138</td>
<td>5</td>
<td>AGATGCTATTCCAACACAGTCCCCAGGGT</td>
<td>Drinkwater et al. 1997</td>
</tr>
</tbody>
</table>

Table 1. Nomenclature, position, primer sequences and references of the used microsatellites.
Molecular characterization of parasite resistant/susceptible Uruguayan Merino lambs

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

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

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

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.

<table>
<thead>
<tr>
<th>STR</th>
<th>Genic differentiation</th>
<th>Genotypic differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p-value</td>
<td>S.E.</td>
</tr>
<tr>
<td>McM214</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>McM357</td>
<td>.0002</td>
<td>.0001</td>
</tr>
<tr>
<td>McM130</td>
<td>.0315</td>
<td>.0015</td>
</tr>
<tr>
<td>CSRD2138</td>
<td>.0392</td>
<td>.00018</td>
</tr>
</tbody>
</table>

Note: S.E. Standard Error

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

<table>
<thead>
<tr>
<th>STR</th>
<th>Statistic test</th>
<th>Population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p-value</td>
<td>S.E.</td>
</tr>
<tr>
<td>McM214</td>
<td>Def. of Heterozygotes</td>
<td>.8742</td>
</tr>
<tr>
<td></td>
<td>Ex. of Heterozygotes</td>
<td>.1767</td>
</tr>
<tr>
<td></td>
<td>Probability test</td>
<td>.1084</td>
</tr>
<tr>
<td>McM357</td>
<td>Def. of Heterozygotes</td>
<td>.1628</td>
</tr>
<tr>
<td></td>
<td>Ex. of Heterozygotes</td>
<td>.8369</td>
</tr>
<tr>
<td></td>
<td>Probability test</td>
<td>.0303</td>
</tr>
<tr>
<td>McM130</td>
<td>Def. of Heterozygotes</td>
<td>.9777</td>
</tr>
<tr>
<td></td>
<td>Ex. of Heterozygotes</td>
<td>.0523</td>
</tr>
<tr>
<td></td>
<td>Probability test</td>
<td>.7293</td>
</tr>
<tr>
<td>CSRD2138</td>
<td>Def. of Heterozygotes</td>
<td>.4450</td>
</tr>
<tr>
<td></td>
<td>Ex. of Heterozygotes</td>
<td>.5657</td>
</tr>
<tr>
<td></td>
<td>Probability test</td>
<td>.8787</td>
</tr>
</tbody>
</table>

Note: S.E. Standard Error. 1Fisher method computed

### Table 5. Observed and expected heterozygosities (Ho, He), Polymorphic Information Content (PIC) and Fixation Indices (F_{IS}) for each STR by subpopulation (Resistant = R, Susceptible = S and Total).

<table>
<thead>
<tr>
<th>STR</th>
<th>McM214</th>
<th>McM357</th>
<th>McM130</th>
<th>CSRD2138</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ho</td>
<td>0.580</td>
<td>0.960</td>
<td>0.770</td>
<td>0.714</td>
</tr>
<tr>
<td>He</td>
<td>0.553</td>
<td>0.839</td>
<td>0.752</td>
<td>0.726</td>
</tr>
<tr>
<td>PIC</td>
<td>0.522</td>
<td>0.809</td>
<td>0.723</td>
<td>0.667</td>
</tr>
<tr>
<td>F_{IS}</td>
<td>-0.049</td>
<td>-0.145</td>
<td>-0.024</td>
<td>-0.015</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>Locus #1</th>
<th>Locus #2</th>
<th>p-value</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistant</td>
<td>McM214</td>
<td>McM357</td>
<td>.0162</td>
<td>.0006</td>
</tr>
<tr>
<td></td>
<td>McM214</td>
<td>McM130</td>
<td>.4279</td>
<td>.0027</td>
</tr>
<tr>
<td></td>
<td>McM357</td>
<td>McM130</td>
<td>.1474</td>
<td>.0020</td>
</tr>
<tr>
<td></td>
<td>McM214</td>
<td>CSRD2138</td>
<td>.3768</td>
<td>.0021</td>
</tr>
<tr>
<td></td>
<td>McM357</td>
<td>CSRD2138</td>
<td>.3220</td>
<td>.0023</td>
</tr>
<tr>
<td></td>
<td>McM130</td>
<td>McM138</td>
<td>.2421</td>
<td>.0021</td>
</tr>
<tr>
<td>Susceptible</td>
<td>McM214</td>
<td>McM357</td>
<td>.5034</td>
<td>.0028</td>
</tr>
<tr>
<td></td>
<td>McM214</td>
<td>McM130</td>
<td>.0038</td>
<td>.0003</td>
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<td></td>
<td>McM357</td>
<td>McM130</td>
<td>.3752</td>
<td>.0029</td>
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<td>CSRD2138</td>
<td>.5967</td>
<td>.0021</td>
</tr>
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<td></td>
<td>McM357</td>
<td>CSRD2138</td>
<td>.2807</td>
<td>.0018</td>
</tr>
<tr>
<td></td>
<td>McM130</td>
<td>CSRD2138</td>
<td>.0312</td>
<td>.0008</td>
</tr>
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</table>

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±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.

Acknowledgements

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Literature Cited

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