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Marker assisted selection in Rasa Aragonesa sheep breed by using a SNP panel for parentage assignment

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Abstract. Accurate pedigree information is an essential tool in genetic breeding programs to ensure the highest rate of genetic gain and allow the management of inbreeding. In this sense, a panel of 192 SNPs, that included 153 SNPs for parentage assignment and 39 functional SNPs, is being used in the selection program for prolificacy of the Cooperative Oviaragon-Grupo Pastores in Rasa Aragonesa. Preselection of some young rams before entering progeny testing is being performed using some of the functional SNPs. For example, the *FecX*^{R} allele of the *BMP15* associated to increased prolificacy, and the *PrnP* genotypes related to scrapie susceptibility. Recently, four SNPs of the *MNTR1A* and *LEPR* genes associated to seasonality traits in Rasa Aragonesa have been included in the panel of 192 SNPs. Because of the increasing demand for hormone-free products and the evolution of European rules and directives towards a reduction, even a complete cessation of use of exogenous hormones leads to search for alternative methods such as the use of genetic markers which would be a powerful tool in selection programs. Therefore, the first objective of this research was to assess how to select some new SNPs for this SNP custom panel. Secondly, this research also aims to validate some putative causal SNPs using the SNP panel as a proof of concept.

Keywords. Rasa Aragonesa – Prolificacy – SNP – Selection.

Sélection assistée par marqueurs chez la race Rasa Aragonesa à l'aide d´un panel de SNPs pour l'assignation de parenté

Résumé. *La précision de l'information du pedigree constitue un outil essentiel dans les programmes de sélection génétique permettant d'assurer un gain génétique plus élevé et de gérer la consanguinité. Dans ce sens, un panel de 192 SNPs dont 153 SNPs d'assignation de parenté et 39 SNPs fonctionnels, a été utilisé dans le programme de sélection sur la prolificité au sein de la race Rasa Aragonesa, mené par la Coopérative Oviaragon-Grupo Pastores. Avant de commencer les tests de descendance, certains jeunes béliers sont présélectionnés à l'aide de certains SNPs fonctionnels notamment, l'allèle FecX*^{R} *du BMP15 associé à une prolificité accrue, et les génotypes PrnP liés à la susceptibilité à la tremblante. Récemment, quatre SNPs de MNTR1A et LEPR associés à des caractères de saisonnalité reproductive chez Rasa Aragonesa, ont été inclus dans le panel de 192 SNPs. En raison de la demande croissante des produits sans hormones et de l'évolution des normes et des directives européennes en vue d'une réduction, voir même une cessation complète de l'utilisation d'hormones exogènes, l'utilisation de marqueurs génétiques pourrait être une alternative et un outil puissant dans les programmes de sélection. Ainsi, l'objectif de cette recherche est d'évaluer comment sélectionner de nouveaux SNPs pour ce panel de SNP et de valider certains SNPs causaux putatifs, en utilisant le panel de SNP comme preuve de concept.*

Mots-clés. *Rasa Aragonesa – Prolificité – SNP – Selection.*

I – Introduction

Rasa Aragonesa is an autochthonous Mediterranean sheep breed from the northeast of Spain, mainly reared in extensive or semi-extensive farming systems and oriented to meat production. Improvement of farms efficiency is possible by genetics, nutrition and management approaches. In this context, the cooperative Oviaragon-Grupo Pastores carries out since 1994 a selection program for prolificacy in Rasa Aragonesa since the number of lambs born per ewe has a key role in the economic profit and viability of these farms. However, new selection objectives, as maternal ability, are being developed to improve the breed whole efficiency.

Accurate pedigree information is an essential tool in genetic breeding programs to ensure accurate estimated breeding values and genetic response and allow the management of inbreeding. However, the proportion of known sires is usually very low in Spanish meat sheep populations, particularly in breeds reared in high mountain areas such as in the Pyrenees. Single nucleotide polymorphisms (SNPs) are now the DNA markers of choice for parentage assignment. In this sense, we developed a SNP assay to be used in some North-Eastern Spanish meat sheep populations for accurate pedigree assignment (Calvo *et al*., 2018a). This panel is composed by 153 SNPs for parentage assignment, and 39 functional SNPs related to prolificacy, seasonality, scrapie resistance, and others. The selection of these SNPs is based on validated SNPs of different traits (*PrnP*, *BMP15*, *GDF9* or *MTNR1A*), or putative functional causal SNPs included in the panel for study and validation. Therefore, the first objective of this research was to show how to select some new SNPs for the SNP panel. Secondly, to validate some putative causal SNPs using the SNP panel as a proof of concept.

II – Material and methods

1. Animal Samples

For the first objective, an experimental population with phenotypic seasonality data was used. Sheep breeds from the Mediterranean area have a seasonality of breeding activity, showing seasonal patterns of oestrous behaviour and ovulation during spring (from March to July). The spring ovulatory activity has heritability and repeatability values of 0.20 and 0.30, respectively (Hanocq *et al*., 1999), but it is only measured in females, is exhibited relatively late in ewe's life and only in some management systems.

Phenotypic seasonality data were obtained from a Rasa Aragonesa sheep flock managed in an experimental farm ("Pardina de Ayés") owned by Oviaragón S.C.L., and described in Martinez-Royo *et al*. (2017). The experimental period extends from January to August of 2012. The flock was composed of 239 ewes in two age groups: young (all: 1.9 years, n=84) and mature (5.2-7.2 years, n=155; 5.5 ± 0.5; mean ± SD) at the beginning of the experiment. Individual live weight (LW) and body condition score (BCS) on a 1 to 5 scale (Russel *et al*., 1969) were assessed every three weeks. These ewes were managed in a single lot and subjected to the same nutrition and environmental conditions as described in Martinez-Royo *et al*. (2017). Three reproductive seasonality traits were considered and described in Martinez-Royo *et al*. (2017). Briefly, the first one was the total days of anoestrus (TDA), based on weekly individual plasma progesterone levels. TDA was the sum of days in anoestrus, considering anoestrus those periods with three or more consecutive progesterone concentrations lower than 0.5 ng/ml. The second reproductive seasonality trait was the progesterone cycling months (P4CM), defined for each ewe as the rate of cycling months based on progesterone determinations. When progesterone level was higher or equal than 0.5 ng/ml in at least one blood sample in that month, the ewe was considered cyclic in that particular month. Finally, the third reproductive seasonality trait considered was the oestrus cycling months (OCM), defined as the rate of months cycling based on daily oestrous records for each ewe. When

at least one oestrus was recorded in that month, an ewe was considered cyclic in that particular month. Eight vasectomised rams fitted with harnesses and marking crayons were joined with the ewes, and daily oestrous detection was performed. Thus, after natural mating, oestrus was recorded as a colour mark on the rump of the ewes.

A total of 3200 ewes from 5 flocks have been genotyped using the SNP panel, mainly for parentage assignment purpose. Phenotypic data related to prolificacy, fertility, seasonality, and the age at first lambing (AFL) were collected. However, we do not have phenotypic data for all animals. Currently, we have data for prolificacy in the whole population and for the age at first lambing. As a proof of concept, we have used a total of 351 ewes phenotyped for AFL. For this phenotype, 191,114 first lambing records (ewes without hormonal treatments) from 327 farms were analyzed using the GLM procedure (SAS) and were corrected for environmental effects. The model included *FecX*^{R} genotype, farm, month and year of the first lambing as fixed effect. Model residuals were used in the association studies.

2. Sampling and genotyping

Only two genes affecting reproductive seasonality traits have been successfully identified, the melatonin receptor subtype 1A (*MTNR1A*) (Pelletier *et al*., 2000; Mura *et al*., 2014), and the arylalkylamine Nacetyltransferase (*AANAT*) (Ding-ping *et al*., 2012). *MTNR1A* has been repeatedly proposed as candidate gene and seems to play a key role in the control of photoperiod-induced seasonality mediated by the circadian concentrations of melatonin (Notter and Cockett., 2005). In this sense, the effects of this gene have been validated in Rasa aragonesa sheep breed (Calvo *et al*., 2018b), and three SNPs of this gene were included in the SNP panel. Moreover, other candidate genes that could be related to seasonality traits were studied. Leptin, a protein secreted mainly by fat tissue, is important in appetite control, energy balance and reproduction (Taheri and Parham., 2016). Leptin´s physiological effects on reproduction including puberty, estrous cycle, pregnancy, lactation, and even the early stages of embryo development have been proven. Polymorphisms in leptin receptor gene (*LEPR*) have been associated with delayed onset of puberty and with decreased ovulation and lambing rates in prolific Davisdale sheep (Juengel *et al*., 2016).

Genomic DNA was extracted from blood samples of 268 ewes (125 and 78 mature and young ewes respectively) from the total ewes of the flock using standard protocols. Some ewes were not considered because of missing data in some variables. For *LEPR* gene, PCR products from exons 4 and 20 of 20 ewes with extreme phenotype values for the TDA and OCM were used to search polymorphisms in the experimental population. Standard protocols for PCR amplification and sequencing were used. The homology searches were performed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). To align the sequences, the CLUSTAL Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) software was used. In addition, the ENSEMBL Variant Effect Predictor (VEP) was used to predict the possible impact of an amino acid substitution. Locations of SNPs were identified based on genome version of Ovis aries Oar_v3.1. Four SNPs related to *LEPR* gene were selected for genotyping the whole population: one in exon 4 (rs411478947) and 3 in exon 20 (rs412929474, rs428867159, and rs405459906). These SNPs were genotyped by Kompetitive allele specific PCR (KASP, LGC, Biotools, Spain) following the manufacter's instructions.

A total of 3200 ewes were genotyped using KASP technology using the panel of 192 SNPs that included 153 SNPs for parentage assignment and 39 functional SNPs. The functional SNPs are located in genes that could be related to prolificacy (*BMP15* and *GDF9*), seasonality (*MTNR1A*, *ANNAT* and *NPSR1*), AFL (*KISS1*, *KISS1R*, *LEPR*, *IGFR1*), and others (*GHR*, *PRNP*, *TMEM154, SPTAN1*). The genotypes of the population of 351 ewes with phenotypic data for AFL were used. Also, the ENSEMBL Variant Effect Predictor (VEP) was used to predict the possible impact of an amino acid substitution. Locations of SNPs were identified based on genome version of Ovis aries Oar_v3.1.

3. Statistical analysis

The Hardy–Weinberg equilibrium exact test values, observed and expected heterozygosities and minor allele frequency (MAF) for each SNP were estimated using PLINK 1.9 software (Purcell *et al*., 2007).

SNP association studies: The association between *LEPR* polymorphisms and the reproductive seasonality traits (TDA, P4CM, and OCM) were performed by fitting a Linear Mixed Model using the MIXED procedure of SAS statistical package. The model included SNPs genotype (S), age (mature and young ewe) (A), and the interaction of age × genotype of the SNPs (A × S), as fixed effects; live weight (LW) and body condition score (BCS) as covariates; and the animal (An) and the residual (e) as random effects. Homogeneous variance for the residual (e ~N (0, ! 2)) was fited. To test differences between genotypes, the least square means (LSMEANS) for each pairwise comparison were estimated. Bonferroni correction was applied to take into account for multiple tests. All SNPs were independently analyzed with the same statistical model.

Haplotype association studies: The gametic linkage disequilibrium (LD) among SNPs (D' and r2) within the *LEPR* was calculated and visualized using the program Haploview v4.2 (Barrett *et al*., 2005). The SNPs rs411478947, rs412929474, rs428867159, and rs405459906 were phased with PLINK1.9 using the expectation–maximisation (E-M) algorithm to assign individual haplotypes. Diplotypes with a posterior probability higher than 0.7 were considered.

Associations between the haplotypes and reproductive seasonality traits were performed using the MIXED procedure of SAS. The model fitted was similar to that used for the SNP association studies, but including the haplotype (H) effect and the interaction age × haplotype (A × H). Haplotypes for each individual were codified as 0, 1 or 2 indicating the copies number of each haplotype. Only haplotypes with a frequency greater or equal than 1% were considered. To test differences between haplotypes, the least square means (LSMEANS) for each pairwise comparison were estimated. Bonferroni correction was applied to take into account for multiple comparisons.

The association analysis was performed with the GCTA software (Yang *et al*., 2011) taking into account the 192 SNPs. Bonferroni correction was applied to take into account for multiple tests.

III – Results and discussion

For the *LEPR*, the entire exon 4 (330 bp) and almost the complete sequence of exon 20 (909 bp: total coding region [828 pb] + partial 3'UTR [81 pb]) were sequenced. Sequences revealed 11 polymorphisms: 3 and 8 SNPs in exons 4 and 20, respectively. All SNPs were in Hardy-Weinberg equilibrium. In exon 4, two synonymous (rs159694506 and rs159694508) and one non-synonymous (rs411478947) polymorphisms were detected. The non-conservative change of an Arginine to Cysteine (Arg62Cys) (rs411478947) was predicted as tolerated but with a low SIFT value of 0.05 by VEP software. In exon 20, five synonymous (rs403654953, rs426037269, rs415715948, rs414501727 and rs427778198) and three non-synonymous (rs412929474, rs428867159 and rs405459906) polymorphisms were detected. SNPs association analysis showed that non-synonymous SNPs in exon 20 were associated to reproductive seasonality traits. The interaction between the SNP and age affected the TDA (P=0.0004), P4CM (P=0.0005) and OMC (P=0.02) traits for SNP rs412929474, showing different effects in mature and young ewes. The SNP rs405459906 was also significant for the interaction SNP x age (P=0.04). After Bonferroni correction only the TDA phenotype differed among genotypes in young ewes (SNP x age was significant), finding significant differences between GG and AG genotypes (P=0.027). Haplotype association studies confirmed the significant SNP x age interactions (Table 1). In this sense, TDA phenotype differed among haplotypes (P<0.05) at young and adult ewes for h1 haplotype, finding significant differences between 0

and 1 copies (P<0.05). However, animals with no copies of h1 had lower TDA value than those with one copy in mature ewes, while in young ewes, animals with no copies of h1 had higher TDA value than those with one copy. This result could indicate either different behaviour depending on age or that this haplotype is in linkage disequilibrium with other SNPs not detected in this study. The haplotypes h2 and h4 were also associated with OMC trait considering the whole population.

Table 1. Type III test for the haplotype and haplotype x age effects of block 5 on the *LEPR* gene using the seasonality phenotype data from Rasa Aragonesa ewes. The least square means and standard errors for the haplotype effect on the *LEPR* gene are also shown. Only significant haplotypes after Bonferroni correction are shown. Different letters indicate significant differences: a, b: P<0.05. SNP Block: rs411478947, rs412929474, rs428867159, and rs405459906

			Haplotype effect[1]				
Trait	**Haplotype**	**Freq.**	**P-value**	**age**	**0 copies**	**1 copy**	**2 copies**
OMC	h2 (GGTG)	0.08	0.0081	total	0.54+0.02a	0.44+0.05b	0.26+0.14ab
OMC	h4 (GGCA)	0.75	0.0067	total	0.40+0.05a	0.53+0.02b	0.56+0.02b
			Haplotype x age effect[1]				
TDA	h1 (GATG)	0.11	<0.0001	mature	55.01+5.18a	85.81+9.07b	88.36+24.97ab
				young	80.30+6.91a	30.03+12.66b	43.98+118.62ab
OMC	h1 (GATG)	0.11	0.0007	mature	0.57+0.02	0.46+0.04	0.41+0.11
				young	0.48+0.03a	0.66+0.06b	0.44+0.20ab

[1] 0 copies: LSMEANS and SE for 0 copies of the haplotype; 1 copy LSMEANS and SE for one copy of the haplotype; and 2 copies: LSMEANS and SE for 2 copies of the haplotype.

These SNPs are included in the SNP panel and will be used for validation studies of the effects found for the SNPs.

Secondly, we tried to validate some putative functional causal SNPs using the SNP panel as a proof of concept. Association studies using GCTA software showed 16 SNPs associated to AFL trait ($p_{nominal} < 0.05$). These SNPs were located in different genes *BMP15*, *KISS1R*, *MTNR1A*, *SPTAN1*, *FA2H*, *TMEM154*, *MTUS1* genes. However, at the genome level ($p_{Bonferroni} < 0.05$) only the SNP rs421419167 (*BMP15*/*FecX*^{R} allele) was significantly associated to AFL. Associations at genome-wise suggestive significance ($p_{suggestive} \leq 1/n$) were found for three SNPs: rs421419167, rs398938610 and rs412567923 located in *BMP15* (*FecX*^{R} allele), *KISS1R* and *SPTAN1* genes, respectively. The *FecX*^{R} allele causes increased prolificacy in heterozygous and sterility in homozygous ewes. The SNP rs398938610 is a non-conservative mutation (p. C309F) located in *KISS1R* gene. This mutation was predicted as tolerated but with a low SIFT value (0.06) by VEP software. Kisspetin and its receptor *(KISS1R)* form a system that regulate the release of GnRH that modulates the release of gonadotropins from the pituitary. *KISS1/KISS1R* system seems to be important for reproductive physiology aspects, ranging from the initiation of puberty to the induction of ovulation. These results have to be validated in a bigger population.

The results provide an additional resource of potential genetic markers for breeding programs considering the size of the effects found and the relative high frequency of some of the favourable alleles in Rasa Aragonesa animals.

IV – Conclusion

In this work, we confirmed the usefulness of a SNP panel composed by 153 SNPs selected for parentage assignment, and 39 functional SNPs related to prolificacy, seasonality, scrapie resistance, and others. We have validated some SNPs associated to different traits in other studies (*BMP15*/*FecX*^{R} allele, *LEPR*) and putative functional causal SNPs (*KISS1R* and *SPTAN1)*. Fur-

thermore, we have assessed the involvement of the *LEPR* gene in reproductive seasonality in ruminants, including some of the *LEPR* SNPs in the SNP custom panel. These polymorphisms could be useful for the breeding program as genetic markers to identify less seasonal or more prolific animals, and to design adequate decisions about its management in the selection program.

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