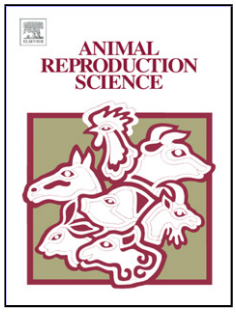

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Characterisation of the Melatonin Receptor 1A (*MTNR1A*) gene in the Rasa Aragonesa sheep breed: association with reproductive seasonality

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ABSTRACT

The ovine *Melatonin Receptor 1A (MTNR1A)* gene was structurally characterised and association between its variants and the reproductive seasonality was examined in a daughter design comprising three families of Rasa Aragonesa sheep breed. Sequencing of six Rasa Aragonesa ewes with extreme values for seasonality trait revealed 28 polymorphisms: 11 SNPs in the coding region (all in exon 2), and 17 SNPs in the promoter region MTNR1A. All the substitutions in the coding region were found most likely lacking any phenotypic effect, because they are conservative mutations or were not part of the transmembrane domain. The silent mutations, which had shown association with reproductive seasonality in other breeds, were also found and genotyped in Rasa Aragonesa. The T allele of SNP606/*RsaI* of MNTR1A gene was associated with a greater percentage of oestrous cyclic ewes in the Rasa Aragonesa breed, indicating that this SNP may be in linkage disequilibrium with a mutation responsible for this trait close to *MTNR1A*, or in regulatory sequences of the gene. In this sense, several SNPs affecting a binding element for some transcription factors have been identified in the promoter region. The SNPs at 422 and 527 positions could constitute a binding element for some transcription factors (TFs), located in an EF2 and SRY consensus sites in the promoter region, respectively. Haplotype h_5 showed significant differences with the h_2 haplotype (66% compared to 49.2%) on oestrous cyclicity, thus these results are consistent with genotypic associations for each SNP. Haplotype with T, A and T alleles for SNPs 422, 677 (promoter region) and 612 (exon 2) showed an increase of the percentage of oestrous cyclic ewes. Although some of these mutations have been associated with seasonal reproduction, further studies with a more appropriate animal design as well as functional studies of TF binding activity are needed.

Keywords: *Ovis aries*, non-breeding, out of season, anoestrus, oestrous cyclicity, *MTNR1A*

1. Introduction

Many sheep breeds from Mediterranean area have seasonal patterns of oestrous behaviour and ovulation. Maximal reproductive activities occur from August to March. This reproductive seasonality induces great variation in lamb production and, therefore, in the market price of lamb meat. Hormonal treatments are widely used in some countries to induce out-of season reproduction, but the increasing demand for free-hormone products leads to a search for alternative methods. In the case of Rasa Aragonesa as in other Mediterranean breeds a percentage of ewes in good management and feeding conditions have spontaneous ovulations in spring, and can be naturally mated throughout the year (Folch et al, 1990**;** Folch and Alabart, 1999). This spring ovulatory activity is under genetic control (Hanocq et al., 1999) but selection for improved fertility in accelerated lambing systems is particularly challenging because of the complexity of the system (Notter 2002). In this context, the use of genetic markers of photoperiod sensitivity is a promising approach to decrease seasonality of reproduction in sheep

The melatonin receptor subtype 1A (MNTR1A) gene has been repeatedly proposed as a candidate gene and seems to play a key role in the control of photoperiod-induced seasonality mediated by the circadian concentrations of melatonin (Dubocovich, 1988; Weaver et al., 1996). Different studies in different sheep breeds have found two silent mutations in positions 606 and 612, associated with the seasonal reproductive trait (Pelletier et al.2000; Notter et al., 2003; Chu et al., 2003; Faigl et al., 2008, Mura et al., 2010; Mateescu et al., 2009; Teyssier et al., 2000; Carcangiu et al., 2009, 2011). However, Hernandez et al. (2005) did not find any relationship between MNTR1A polymorphisms and reproductive seasonality in Ile-de France ewes, indicating that the effect of these polymorphisms could depend on the breed and/or environmental conditions.

In the present research, the association between MNTR1A polymorphisms and reproductive seasonality were investigated in a daughter design comprising three families of Rasa Aragonesa breed. For this purpose, detection and characterisation of polymorphisms were performed in the whole coding region and promoter of the MTNR1A gene in Rasa Aragonesa ewes. Secondly, an association study between some of the polymorphisms and reproductive seasonality was performed.

2. Materials and methods

All experimental procedures were performed in accordance with the guidelines of the European Union (2003/65/CE) and Spanish regulations (RD 1201/2005, BOE 252/34367–91) for the use and care of animals in research.

2.1. Animals and experimental design

This experiment was conducted from January to August in the facilities of Centro de Investigación y Tecnología Agroalimentaria (CITA), a research Centre located in Zaragoza (Spain). Rasa Aragonesa is an autochthonous Mediterranean breed of sheep from the northeast of Spain, with about 500,000 animals recorded, mainly reared in extensive or semi-extensive farming systems and oriented to meat production. A total of 80 single reared ewes from a daughter design comprising three sire families of the Rasa Aragonesa sheep breed (26, 25 and 29 animals per family represented by rams A, B and C, respectively) were used. Sires heterozygous at position 606 and 612 were chosen. The ewes have born from January to April and came from 21 different farms. Ewes from each ram were selected to be as unrelated as possible on the basis of pedigree information. The age of all ewes was 3 years old, with similar body conditions (ranging from 2.5 to 3.0 of 5.0) and were maintained under the same management and fed *ad libitum* while grazing pasture. Weight and body condition score were recorded weekly from the beginning of the experiment and

measured according to Russel et al. (1969) by two trained technicians. No hormonal treatments were applied to ewes during the study.

From January to August 2009, four vasectomised rams fitted with harnesses and marking crayons were mixed with the ewes and daily oestrous detection was performed (Radford et al. 1960). Thus, after natural mating, oestrus was recorded as a colour mark on the rump of the ewes, easy to identify visually. As ovulatory cycles occur approximately every 17 days, the colour of the marker crayon was changed every 2 weeks to avoid confusing marks between consecutive oestrus.

2.2. Structural characterisation of the MNTR1A gene

Genomic DNA was extracted from ovine lymphocytes according to the salting-out procedure described by Miller et al. (1988). Primers designed from sheep sequences NM_001009725 and AF078545 were used to amplify total coding and 5' UTR regions and partial 3' UTR and promoter genomic regions of the MNTR1A gene (Table 1). Genomic DNA (50 ng) of six Rasa aragonesa ewes with extreme values for the reproductive seasonality trait and the three rams was amplified in a final PCR volume of 25 µl containing 5 pmol of each primer, 200 nM dNTPs, 2.0 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100 and 0.75 U Taq polymerase (Taq, Biotools). Standard amplification cycles were used. The PCR products were sequenced using an ABI Prism 3700 (Applied Biosystems) and standard protocols. Direct sequencing of the PCR products of a small sample of ewes were used to search polymorphism in the experimental population (two ewes for each family) with extreme values for seasonality trait was performed as above, and standard protocols were used to search for polymorphisms. Homology searches were performed using the BLAST algorithm (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/BLAST/). Sequence alignments were performed using

CLUSTALW software (http://www.ebi.ac.uk/clustalw/). Studies of putative regulatory elements within the promoter were performed using TF Search (http://www.cbrc.jp/research/db/TFSEARCH.html) and Signal Scan (http://www-bimas.cit.nih.gov/molbio/signal/) software.

MNTR1A genotyping was performed in the 80 ewes and the three sires for the SNPs at positions 606 and 612 (according to GenBank reference sequence U14109), and the SNPs in the promoter region. A fragment of Exon 2 was amplified containing the two SNPs at positions 606 and 612 (Table 1). PCR product was digested separately for SNPs 606 and 612 with *RsaI* and *MnlI* restriction enzymes, respectively.

Because of the large number of SNPs found in the promoter of *MTNR1A*, a PCR comprising all promoter polymorphisms was designed and amplification products were sequenced with forward primer (Table 1).

2.3. Statistical analyses

Oestrous cyclic and acyclic ewes in a given month were coded with "1" and "0", respectively. A ewe was considered oestrous cyclic when at least one mark was detected in that month. All ewes, except one that was excluded from the study, had at least one oestrous record between February and July. In January and August all ewes were oestrous cyclic and then these months were excluded from the analysis. To study genotypic association between each SNP and the oestrous activity, the percentage of oestrous cyclic ewes between February and July was analysed using repeated-measures GLM procedures for dichotomous variables (Guthrie, 1981) using the CATMOD procedure of SAS (SAS Institute Inc., 2004). The model used was: FC (ik) = μ + Genotype (i) + Month (k) + GxM(ik), where FC(ik) is the frequency of oestrous cyclic ewes with genotype(i) at Month(k); μ is the overall mean of the frequency of oestrous cyclic ewes from February to July; Genotype(i) is the effect of genotype(i) nested to ram;

Month (k) is the effect of the month (February, March, April, May, June and July); and GxM (ik) is the interaction effect of Genotype x Month.

Haplotype inference was performed using PHASE program SeqPHASE step 1 (Flot 2010) was used to generate PHASE input files from FASTA alignments. The inference of haplotypes from genotype data was performed with PHASE 2.1, using the Bayesian statistical method and a modelling option that allows for recombination and decay of Linkage Disequilibrium (LD) with distance (Stephens et al. 2001; Stephens and Donnelly 2003). Finally, SeqPHASE step 2 was used to convert PHASE output files back into FASTA alignments. All probabilities of ambiguous phases were taken into account. Nevertheless, as indicated by Lake et al. (2003) haplotype frequencies should be at least 5% to avoid biased estimations of the regression parameters, haplotypes with frequencies below 5% were pooled together into a rare haplotype group (h_r; Oh et al. 2007). Association of the inferred haplotypes with the percentage of oestrous cyclic ewes from February to July was analysed by a logistic regression model without intercept, using PROC LOGISTIC of SAS (SAS Institute Inc., 2004). The model included the haplotypes and the interactions between haplotypes corresponding to haplotype combinations (diplotypes) with frequencies of at least 5%. Model fitting was assessed by the Likelihood Ratio and the Hosmer and Lemeshow goodness-of-fit tests. Deviation from binomial variability (over/under-dispersion) was evaluated using the ratios of the Deviance and Pearson chi-square to the number of the degrees of freedom. As the inferred haplotypes were not in Hardy-Weinberg equilibrium (HWE), a direct design matrix (DDM; Oh et al. 2007) was used to avoid biases that occur when modelling with an indirect design matrix under departure from HWE (Satten and Epstein, 2004). The most frequent haplotype was used as baseline to compare the remainder haplotyes.

3. Results

3.1. Structural characterization

The entire Exon 1, and 824 bp and 1526 bp of the Exon 2 and promoter region respectively, were characterised. Exons were identified by comparison with ovine sequence (GenBank sequence NM_001009725 and AF078545). Sequencing of the six Rasa Aragonesa ewes with extreme values for the reproductive seasonality trait revealed 28 polymorphisms: 11 SNPs in the coding region (all in Exon 2), and 17 SNPs in the promoter region (Figure 1). In the coding region, 7, 2 and 2 synonymous were identified, conservative and non-conservative amino acid substitutions, respectively (Figure 1). Additionally, cis-regulatory elements in the 5' flanking region were identified with the informatics programs TFSEARCH and Signal Scan, which predicted several putative binding sites for various trans-acting factors. Seven polymorphisms detected in the 5' flanking region were located within possible trans-acting factor binding sites. For instance, the SNPs at positions 422 (C/T) and 423 (G/T) are present in the EF2 consensus site, the SNPs at 436 (C/T), 527 (A/G) and 686 (T/C) are present in the SRY site, the SNP at position 482 (T/C) is in a Nkx-2 motif and an additional polymorphisms was found at position 750 (G/A) affecting a Brn-2 and Oct -1 sites, according to GenBank reference sequence AY524665.

3.2. Genotypic association studies

The percentage of cyclic ewes decreased between February and April, then increased to 100% in August (Fig.2). Body weight and condition were not significantly different during the experiment.

Because of the mutations that led to an amino acid change are not part of the transmembrane domains, only SNPs at positions 606 and 612 in Exon 2, that have been

199 associated to seasonal reproduction in other breeds, and SNPs in promoter were
200 genotyped for association studies.

201 For SNPs at positions 606 and 612, a PCR product of 250 bp was amplified for all
202 samples. Enzyme digestion using *RsaI* for SNP 606 produced three (148, 79 and 23 bp)
203 and two (148 and 102 bp) bands for the C (R) and T (r) alleles, respectively.
204 Electrophoresis analysis of *MnlI* digestion produced two (237 and 13 bp) and three (170,
205 67 and 13 bp) bands for the G (+) and A (-) alleles, respectively. The three rams were
206 heterozygous for SNPs 606 and 612. Rasa aragonesa showed similar frequencies for
207 alleles at 606 position (p(T)= 0.51) and a greater frequency of G (+) allele (p(G) = 0.87)
208 at position 612. Genotype distribution was in Hardy- Weinberg equilibrium for the two
209 SNPs.

210 All terms of the model were highly significant for SNPs 606 and 612. Association
211 studies (Table 2) showed a significant effect of the genotype nested to ram at position
212 606 (*RsaI*) on cyclicity. In across-family analysis the percentage of oestrous cyclic ewes
213 was 21.9% greater in TT homozygous than in CC (77.5% compared to 55.6%, P <0.01).
214 Heterozygous ewes showed intermediate values (67.1%) not significantly different from
215 TT or CC genotypes. In within-family analysis, genotype was only significant for the
216 reproductive seasonality trait in family A. However, the three families showed the same
217 tendency, with the TT genotype associated to out-of season breeding ability. Significant
218 differences in oestrous cyclicity among months and genotypes for SNP606 were found.
219 The greatest differences between TT and CC genotypes in the percentage of oestrous
220 cyclic ewes were reached in May (27.8%, P < 0.1), June (29.4%, P <0.05) and July
221 (28.9%, P <0.05) (Figure 2). The same patterns were found when analysis was
222 conducted across families (Figure 2a) or within families (Figure 2b). For the SNP612
223 (*MnlI*), no significant differences were found between GG and GA genotypes in either
224 across-family or within-family analyses (Table 2). Significant differences favouring AA

over both GG and GA genotypes were found in across-family and within-family (ram “B”) studies. However, these significant results were due to the unique ewe with AA genotype present in this study, belonging to the Ram “B” family, which was oestrous cycling throughout all the study period. As expected, when removing this ewe from the analysis, the significant differences between genotypes disappeared. Likewise, no significant differences in oestrous cyclicity between GG and AG genotypes were found for any particular month when the AA ewe was removed from the analysis.

In the promoter region, 17 SNPs were found but only the Ram "A" was heterozygous for some of the SNPs found, in particular for SNPs 341, 422, 527, 677 and 895, while families “B” and “C” were homozygous for all SNPs. Association analysis showed significant associations for the five polymorphisms. Table 3 shows the percentage of oestrous cyclic ewes according to the genotype for the SNPs in the promoter region.

3.3. Haplotypic association studies

The phased haplotype analysis was conducted on SNP-set containing 7 SNPs: 341, 422, 527, 677, 895 from the promoter region, and 606 and 612 from Exon 2. The three rams were heterozygous for haplotype combinations (diplotype), and then all ewes were considered in the analysis. The haplotype analysis identified 23 putative haplotypes. Only five haplotypes had a frequency of greater than 5%. The model fit was good as evidenced by both, the significant Likelihood ratio (74.4; d.f.=13; $P < 0.0001$) and the non-significant Hosmer and Lemeshow chi-square (2.1; d.f.=7; $P < 0.95$) tests. There was no evidence of over/under-dispersion using either Deviance (21.1; d.f.=16; $P < 0.1759$) or Pearson chi-square (20.2; d.f.=16; $P < 0.2108$) tests, that were very similar. None of the haplotype interactions considered was significant. Haplotype h_5 showed differences ($p = 0.007$) with the h_2 haplotype (66% compared to 49.2% on oestrous

cyclicity) (Table 4). Haplotype h_5 was associated with an increase of the percentage of oestrous cyclic ewes.

4. Discussion

A small sample of ewes with extreme values for the seasonality reproductve trait were used to search for polymorphisms. Using extreme animals to search for polymorphisms increased the probability of detecting polymorphic SNP associated to the trait, but reduced the probability of detecting other polymorphic SNP. Nevertheless, other polymorphisms may exist and could not be found using this small sample of ewes. Eleven SNPs were found in the coding region of the MNTR1A gene. Four of the SNPs had not been previously described in other sheep breeds, with two of these being silent mutations, one a conservative change of a Serine to Asparagine (S112N) and the last one a non-conservative change of a Serine to Proline (S113P). Two amino acid changes previously described in Sarda and Merino d'Arles (V220I and A282D) were also found in the Rasa Aragonesa sheep breed (Pelletier et al., 2000; Carcangiu et al., 2009). Carcangiu et al. (2009) indicated that the amino acid substitutions were not part of the transmembrane domain of the melatonin 1 receptor and, in accordance with Barrett et al. (2003), these changes in amino acids should not modify the receptor's functionality. In the same way, the amino acid substitutions S112N and S113P found in Rasa Aragonesa were not part of the transmembrane domain. Furthermore, all substitutions were found most likely lacking any phenotypic effect using PolyPhen software (http://genetics.bwh.harvard.edu/pph/). The silent mutations, which despite being synonymous had shown association with reproductive seasonality in other breeds (Pelletier et al.2000; Notter et al., 2003; Chu et al., 2003; Faigl et al., 2008, Mura et al., 2009; Mateescu et al., 2009, Carangiu et al., 2009, 2011) were also found in Rasa Aragonesa (at positions 606 and 612 according to GenBank reference sequence U14109). Genotype frequencies for allele

T at positions 606 and G at position 612 were greater than in other breeds (Notter and Cockett, 2005, Chu et al., 2006, Carcangiu et al., 2009).

The oestrous activity of the 80 ewes, as detected using vasectomised rams fitted with harnesses and marking crayons, was similar to that described in the Rasa Aragonesa breed when maintained in pens (Forcada et al., 1992) or in similar conditions as the present experiment (Folch and Alabart, 1999). The present results are in agreement with other studies in that MNTR1A gene is associated with reproductive seasonality. However, most studies concluded that ewes of GG (++) for SNP612 genotypes showed more oestrus in spring (Pelletier et al.2000; Notter and Cocket., 2005; Chu et al., 2006; Faigl et al., 2008, Mura et al., 2009; Carcangiu et al., 2009) while in Rasa Aragonesa sheep breed this SNP was not consistent associated to seasonality trait. Furthermore, results of the present study showed that ewes of TT genotype for SNP606 showed more oestrous activity during the anoestrous season while in the studies of Chu et al. (2006) and Carcangiu et al. (2009) the CC genotype was associated to an increased oestrous activity. These findings, together with the knowledge that these polymorphisms were not the causative mutation, indicate that other genes closely linked or regulatory sequences of the MNTR1A gene could be influencing the ability to breed out of season.

In this sense, when the promoter region of the gene was isolated and characterised, a total of 17 SNPs have been found, showing that the heterozygous genotype of all SNPs was associated to an increase of the percentage of oestrous cyclic ewes. However, this association may be spurious as it relies upon an unbalanced distribution of genotypes and a small number of animals (n = 26) for each SNP. Haplotype analysis results were consistent with those of the genotypic association between each SNP and the oestrus activity. Haplotype h_5 showed significant differences with the h_2 haplotype (P = 0.007). Haplotypes with the T allele at position 606 in Exon 2

(h_3, h_4 and h_5) tended to have more oestrus in spring than haplotypes with the C allele (h_1 and h_2). In the same way, haplotytes with the T-A alleles at SNPs422-677 (h_4 and h_5) also showed an increase of the percentage of oestrous cyclic ewes than the C-G alleles (h_1, h_2 and h_3). Haplotypes with T, A and T alleles for SNPs 422, 677 and 612 (Exon 2) (h_5 = G**T**A**A**A**T**G), respectively, seems to be associated to an increased oestrous activity (Table 4). Only the SNP at 422 position could be the causative mutation. This SNP modify a putative binding element for EF2 transcription factor. The E2Fs regulate the timely expression of a series of genes whose products are essential for cell proliferation (Helin, 1998). Although elongation factors (EF) have been reported to modulate GPCR function by direct interaction with the receptor (McClatchy et al., 2002; Cho et al., 2003), interactions between EF and *GPCRs* genes, such as *MTNR1A,* have not been described. The functionality of these SNPs could be elucidated in further studies by analysing its TF binding activity by band shift or luciferase reporter assays.

Present data, thus, should be considered as preliminary and further studies are needed to evaluate these possible associations more effectively, allowing for distinguishing between three possible explanations: false positive associations, causal effects of SNPs in the promoter region of *MNTR1A* and effects of linked genes.

5. Conclusions

We have demonstrated that the T allele of SNP606 of MNTR1A gene is associated with a greater percentage of oestrous cyclic ewes in the Rasa Aragonesa breed, indicating that this SNP may be in linkage disequilibrium with a mutation responsible for this trait close to *MTNR1A*, or in regulatory sequences of the gene. In this sense, several SNPs affecting a binding element for some transcription factors have been identified in the promoter region. Haplotype h_5 showed significant differences with the h_2 haplotype (66 vs 49.2% on oestrous cyclicity), being these results consistent with

genotypic associations for each SNP. Haplotype with T, A and T alleles for SNPs 422, 677 and 612 showed an increase of the percentage of oestrous cyclic ewes. Only the SNP at 422 position could be the causative. Even though some of these mutations have been associated with seasonal reproduction, further studies with a more appropriate animal design as well as functional studies of TF binding activity are needed.

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Fig. 1. Schematic representation of ovine *MNTR1A*. Boxes represent the exons (coding and non-coding sequences in black and grey, respectively). Polymorphisms are indicated with arrows. The nucleotide positions of the polymorphisms are indicated according to GenBank sequences AY525665 and U14109 for and promoter, Exon 1, and Exon 2 (in brackets), respectively. The amino acid position of the polymorphisms and the amino acid change is indicated according to Uniprot reference sequence P48040. Black arrows show new polymorphisms.

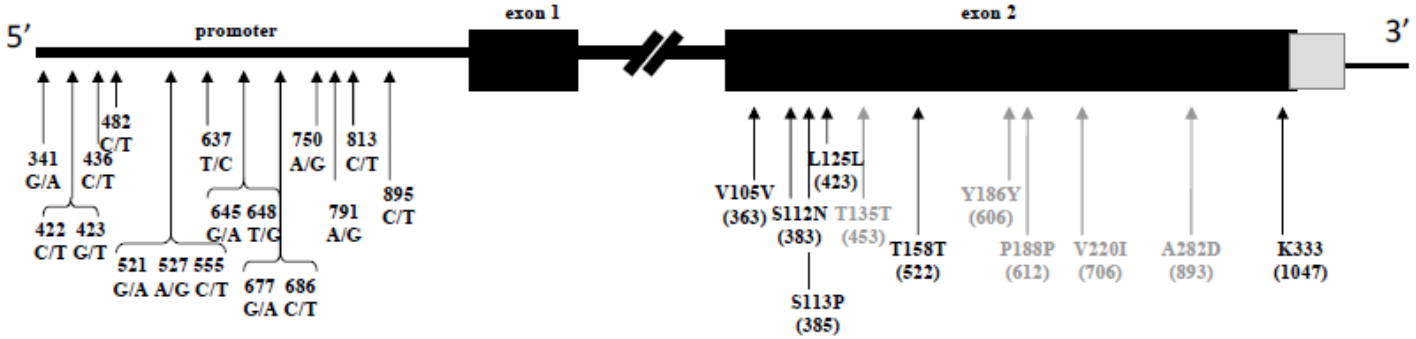
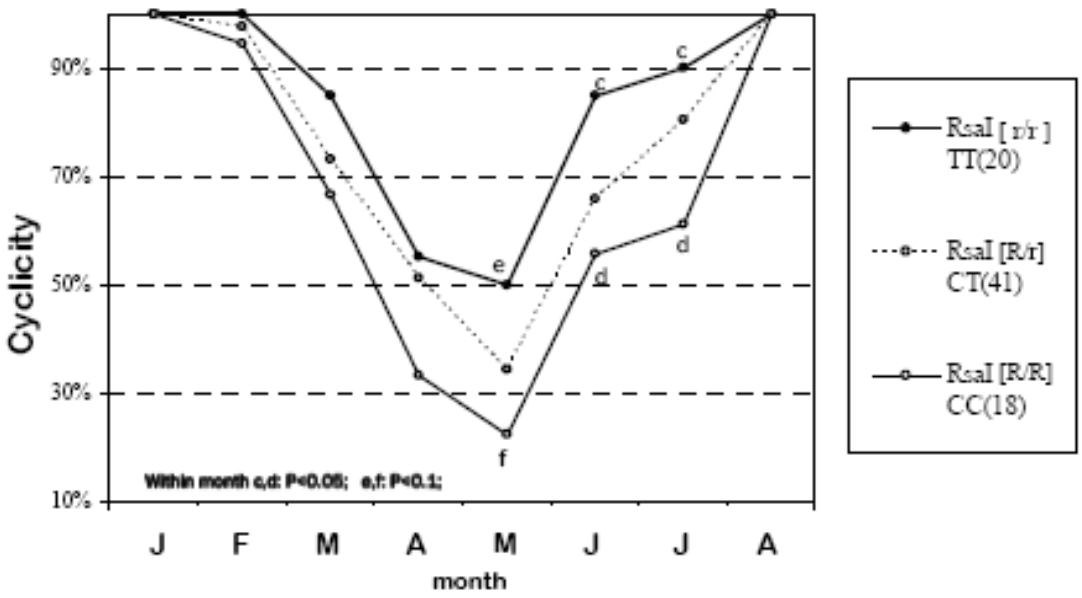


Fig. 2. Percentage differences with oestrous cyclicity across months and genotypes across families (a) and within families (b).

a)



b)

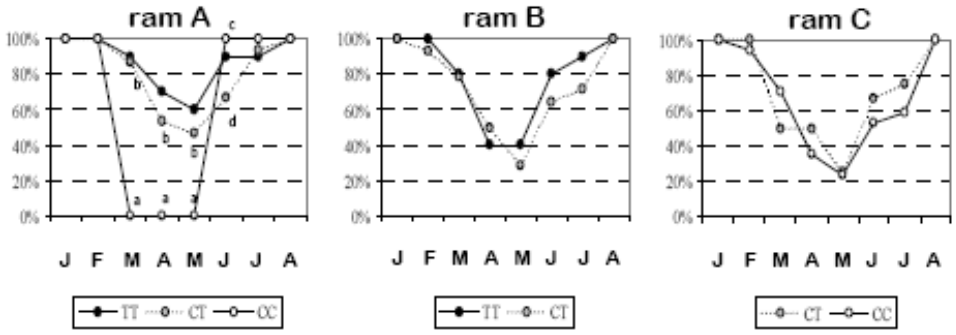


Table 1. Primer sequences and GenBank reference information. PCRs 3 and 6 were used to genotype the SNPs for promoter and exon 2, respectively.

PCR	Primer sequence (5' → 3')	Site	GenBank	Tª ann (ºC)	Size (bp)
1	GCACAAAAAGAAGCCAAGGA	Promoter	AY524665	61	
	TCAGGTGTCGCACTGTAACC	Promoter			777
2	TGTTCCTGGGAGAAGTCTGG	Promoter	AY524665	57	
	CATGCATCAAACCTGGACTG	Promoter			721
3	AGCCTGGTGGCTGGTAGTTA	Promoter	AY524665	59	
	TTTAATGGAGCATGGAAGGG	Promoter			733
4	CTCGACGCTCTGGGGAT	promoter	AY524665	53	
	GCGTTCCTCAGCTTCTTGTT	intron 1			251

5	TGTGTTTGTGGTGAGCCTGG	exon2	NM_001009725	57ºC	
	ATGGAGAGGGTTTGCGTTTA	exon2			824
6	TCCCTCTGCTACGTGTTCCT	exon2	NM_001009725	58	
	GTTTGTTGTCCGGTTTCACC	exon2			250

Table 2. Mean percentage of cyclic ewes from February to July (anoestrous period) according to the genotype for the SNP606 and SNP612 across families and within families. Number of ewes is indicated within parentheses.

	Across families	ram A	ram B	ram C
Genotype *SNP606*				
TT	77.5 (20) a	83.3 (10) a	71.7 (10) a	----- (0)
CT	67.1 (41) ab	74.4 (15) ab	64.3 (14) a*	61.1 (12) a
CC	55.6 (18) b	50.0 (1) b	----- (0)	55.9 (17) a
Genotype *SNP612*				
GG	67.8 (60) b	78.6 (21) a	65.1 (21) b	58.3 (18) a
GA	63.0 (18) b	70.0 (5) a	74.9 (2) b	57.6 (11) a
AA	100.0 (1) a	----- (0)	100.0 (1) a	----- (0)

*One female was excluded from the association study due to the lack of cyclicity in any month, from January to August

a, b: P <0.01.

Table 4. Association analysis of the inferred haplotypes with the mean percentage of cyclic ewes from February to July (anoestrous period) according to the haplotype for the SNPs 341, 422, 527, 677, 895 from the promoter region (GenBank AY524665), and SNPs 606 and 612 from exon 2 (GenBank U14109). Haplotypes with frequencies below 5% were pooled together into a rare haplotype group (h_r). The most frequent haplotype (h_5) was used as baseline to compare the remainder haplotyes.

ID number	Haplotype	Frequency (%)	ln OR	% Cyclic	OR	Significance
h_1	GCAGACA	6.96	-0.20 ± 0.69	47.5	0.47	NS
h_2	GCAGACG	28.75	-0.06 ± 0.35	49.2	0.50	0.007
h_r	XXXXXXX	16.14	0.25 ± 0.53	53.1	0.58	NS
h_3	GCAGATG	6.16	0.71 ± 0.84	58.8	0.73	NS
h_4	ATGAGTG	12.58	0.75 ± 0.48	59.3	0.75	NS
h_5	GTAAATG	29.41	1.33 ± 0.38	66.0	1	---

Table 3. Mean percentage of cyclic ewes from February to July (anoestrous period) according to the genotype for the SNPs in promoter region in family "A" (the position of the SNP is indicated according to GenBank AY524665). Number of ewes are indicated within parentheses.

SNP	Genotype	Cyclicity %
341	GG	52.4 (21)[a]
	AG	73.3 (5)[b]
	AA	--- (0)
422	CC	45.5 (11) [a]
	CT	66.7 (14) [b]
	TT	33.3 (1) [a]
527	AA	50.0 (18) [a]
	AG	70.8 (8) [b]
	GG	--- (0)

SNP	Genotype	Cyclicity %
677	GG	48.6 (12) [a]
	AG	65.4 (13) [b]
	AA	33.3 (1) [a]
895	AA	50.8 (20) [a]
	AG	75.0 (6) [b]
	GG	---(0)

a, b: P <0.01.