The high prolificacy of D'man sheep is associated with the segregation of the *FecL^L* mutation in the *B4GALNT2* gene

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Mutations in the *FecL* locus are associated with large variation in ovulation rate and litter size in the French Lacaune sheep breed. It has been shown that the *B4GALNT2* gene within the *FecL* locus is most likely responsible for the high fecundity in the French breed. In this study, we have highlighted the segregation of the $FecL^L$ mutation within the *B4GALNT2* gene in North African sheep breeds and notably in the highly prolific D'man breed. Genotyping of a sample of 183 Tunisian D'man individuals revealed a high frequency (0.65) of the prolific allele $FecL^L$ which was attributed to the adoption of a decades-old breeding strategy based on the selection of ewe lambs born from large litter size. Homozygous *LL* ewes showed a significantly increased litter size compared to heterozygous and non-carrier ewes ($FecL^L/FecL^L$ =2.47 ± 0.09 versus $FecL^L/FecL^+$ =2.23 ± 0.09, $P < 0.05$ and $FecL^+/FecL^+$ =1.93 ± 0.18, $P < 0.01$). The presence of the $FecL^L$ polymorphism in both D'man and Lacaune breeds argues for an ancestral origin of this mutation and brings an answer to the old question of the genetic determinism of the extreme prolificacy of the D'man ewes. The results of this study can help to establish planned genotype-based mating allowing both higher profit for the breeders and an optimal management of the $FecL^L$ mutation in D'man sheep populations.

Keywords : D'man sheep - Prolificacy - Major gene - B4GALNT2

1 INTRODUCTION

The D'man sheep is a highly prolific breed that spreads in southwestern oases of Algeria (Boubekeur et al., 2015) and south of Morocco (Boujenane, 1999). This breed was established in Tunisia in 1994 from a Moroccan founder flock and expanded widely in the country, particularly in the Tunisian oases (Rekik et al., 2005). A limited number of studies tried to shed light on the genetic determinism underlying the high prolificacy within the D'man breed. The conclusions were never in favor of the segregation of a major gene controlling this trait as it was seen in Merinos Booroola (Bradford et al., 1989; Boujenane et al., 1991; Piper and Bindon, 1985). Moreover, none of the studies made at the molecular level succeeded in identifying genes associated with ovulation rate or litter size (LS). For instance, Vacca et al., (2010) did not find any polymorphisms in seven genotyped mutations located in three previously described prolificacy major genes (*BMPR1B*, *BMP15* and *GDF9*) within a sample of 47 D'man individuals. Another study carried out by Aherrahrou et al., (2015) has found difference in expression levels of the luteinizing hormone β subunit gene (*LHB*) between D'man and other normal prolific local breeds in Morocco. Knowing the role of this gene in the ovulation process, they suggested that the *LHB* gene might be a good candidate for prolificacy variation in D'man. However, these authors did not specify whether they found or not differences in expression levels or polymorphisms between highly and normal prolific D'man ewes.

Recently, we have performed a genetic variant data mining focused on the four prolificacy major gene loci (*B4GALNT2*, *BMP15*, *BMPR1B* and *GDF9*; Vinet et al., 2012; Abdoli et al., 2016) from the comparative analysis of whole ovine genome sequence data publicly available from the European project NextGen (http://projects.ensembl.org/nextgen/). Notably, we have highlighted the presence of a previously described polymorphism in the intron 7 of the *B4GALNT2* gene at the prolific *FecL* locus (OAR 11; Drouilhet et al., 2013) in a subset of Moroccan sheep individuals within the D'man, Sardi, Beni Guil and some other local breeds. The prolific allele, named $FecL^L$ was originally shown to have large effects on ovulation rate and LS in the French Lacaune meat breed (Bodin et al., 2002, Drouilhet et al., 2009; Martin et al., 2014). At the molecular level, the $FecL^L$ mutation leads to a huge increase

of the *B4GALNT2* expression and glycosylation activity within the ovarian granulosa cells targeting the inhibin/activin system. This glycosylation mechanism was proposed as the physiological determinant of the regulation of ovulation rate and LS in the Lacaune breed (Drouilhet et al., 2013). The main purpose of the present study was to validate the presence of the polymorphism detected within the *B4GALNT2* gene at the *FecL* locus in a larger number of D'man animals originated from Tunisia and to investigate its association with prolificacy (measured through LS) in order to check that the mutation has also an effect on the D'man's genetic background.

2 MATERIALS AND METHODS

2.1 Ensembl/NextGen data mining

We have looked for previously identified SNP and small insertions/deletions in the sheep genome v3.1 using the Ensembl genome browser (www.ensembl.org, accessed January 2016) at the four known prolificacy major loci, *B4GALNT2* (11: 36,929,322-36,992,982), *BMP15* (X: 50,970,938-50,977,454), *BMPR1B* (6: 29,361,947-29,448,079) and *GDF9* (5: 41,841,034-41,843,517). We have focused on the alleles named $FecL^{L}$ in *B4GALNT2*, $FecX^{I, H, B, G, L, R, Gr, O, Bar}$ in *BMP15*, $FecB^{B}$ in *BMPR1B* and $FecG^{H, T, V, E, WN}$ in *GDF9* that were shown to affect prolificacy in various sheep breeds (reviewed in Vinet et al., 2012; Abdoli et al., 2016, genomic coordinates in Table 1). At each position we have extracted the population information data coming from the whole genome sequencing publicly available from the NextGen project (http://projects.ensembl.org/nextgen/).

2.2 Animal sampling

Jugular vein blood samples (5 ml per animal) were obtained from 152 D'man ewes and 31 rams in eleven different flocks spread across Tunisia by persons qualified in animal experimentation (European standards) and under the Tunisian Veterinary Authorities' rules. The flocks were ranked into three rearing mode (RM) categories corresponding to commercial private (RM1; 50% of the animals), state (RM2; 15% of the animals) and small familiar farms (RM3; 35% of the animals). Most of the individuals (91%) have at least 3 consecutive lambing records (Figure 1).

2.3 Polymorphism genotyping assays

At the *B4GALNT2* locus, the genotyping of *FecL*^{L} (*B4GALNT2* intron 7, OAR11:36938224T>A, NC_019468.1, Drouilhet et al., 2013) was obtained by a first step of KAPA Blood PCR amplification of a specific fragment encompassing the mutation position (KAPA Biosystems) and a second step of fluorescent Kompetitive Allele Specific PCR via the KASP V4.0 2x Master mix (LGC genomics) as already described (Chantepie et al., 2018). KAPA Blood PCR amplification was also used for subsequent DNA sequencing as described (Talebi et al., 2018) of exons 1 and 2 of *BMP15* and *GDF9*, and exon 7 of *BMPR1B*. Obtained reads were aligned against the ovine reference sequence for each gene of interest (ovine reference genome v3.1; *BMP15*: NC_019484.1; *GDF9*: NC_019462.1 and *BMPR1B*: NC_019463.1) using CLC Main Workbench Version 7.6.4 (www.clcbio.com) in order to assess for polymorphisms. All primers used for genotyping are shown in Table 2.

2.4 Statistical analyses

The genotypic frequencies at the *FecL* locus were tested for deviation from Hardy-Weinberg equilibrium according to a Chi-square (χ^2) test and level of significance at 1 degree of freedom. The association between *FecL* genotypes and LS was analyzed using a two-way analysis of variance (ANOVA) followed by post hoc Tukey's multiple comparisons test. The ANOVA model used was: $LS_{ij}= \mu + RM_i+G_j + e_{ij}$, where LS denotes the mean litter size, μ the overall sample mean, RM_i the fixed effect of the rearing mode (i=1, 2, 3), G_j the fixed effect of the genotype (j=++, *L+, LL*) and e_{ij} is the random error. The association between genotypes at *FecX*, *FecG* and *FecB* loci and LS of *FecL* +/+ ewes was analyzed using a one-way ANOVA in order to test only for the genotype effect. A significant difference was considered when the P-value $P < 0.05$.

3 RESULTS

A data mining approach aimed at identifying genomic variants (SNP and small Indels) recorded in the Ensembl database (www.ensembl.org) was performed at the four loci presently known to affect ovulation rate in sheep. We have particularly focused on the positions of the sixteen proven prolific alleles within the four following genes: *B4GALNT2, BMP15, BMPR1B* and *GDF9*. As shown in Table 1, only the *FecL*^{L} prolific allele (OAR11: 36938224A) within the *B4GALNT2* gene was detected

(0.18 in frequency among 160 individuals) in a subset of Moroccan sheep (MOOA) from several breeds whose genome was sequenced in the frame of the NextGen project (http://projects.ensembl.org/nextgen/). When looking more specifically at the Moroccan breeds affected, we have highlighted the segregation of the $FecL^L$ allele in D'man, Sardi, Beni Guil and some other local breeds (supplementary Table S1). Notably, the well-known prolific D'man breed exhibited a very high frequency (0.58) of the $FecL^L$ allele (23 carriers among the 30 randomly chosen animals). The apparent frequency appeared lower in Sardi (4 heterozygous among 27 animals), in Beni Guil (2 heterozygous among 6 animals) and in a mix of local populations (15 carriers, including 2 homozygous, among 72 animals).

In order to specifically confirm the segregation of the $FecL^L$ allele and its effect on prolificacy in the D'man breed, we have genotyped 183 individuals (31 males and 152 females with litter size records) at the SNP present in the intron 7 of *B4GALNT2* (OAR11:36938224T>A) in the Tunisian D'man sheep population. As shown in Table 3, we have confirmed the segregation of the $FecL^L$ prolific allele in this D'man sheep population. Observed genotype frequencies in the whole sampled animals were 43.2 %, 44.3 % and 12.5 %, for *LL*, *L*+ and ++ genotypes, respectively (Table 3), and in accordance with the Hardy-Weinberg equilibrium (χ^2=0.10, P = 0.75). Genotype frequencies of homozygous individuals varied widely between RM1/RM3 and RM2 flocks with values ranging from 5.5 % (RM1) to 41 % (RM2) for ++ individuals and from 18 % (RM2) to 48.5% (RM1) for *LL* animals (Table 3). Genotype of the ewe (P = 0.0023) and rearing mode (P = 0.044) had a significant effect on litter size (LS) with no interaction between the two parameters. Average LS was 1.93 ± 0.18, 2.23 ± 0.09 and 2.47 ± 0.09 for ++, *L*+ and *LL* ewes, respectively (Table 4). Tukey test showed that mean LS was significantly higher in ewes with *LL* genotype than in those with ++ (P < 0.01) and *L*+ genotypes (P < 0.05). However, the increased LS of *L*+ ewes compared to ++ was not significant (P = 0.21) surely due to the low number of ++ individuals (n=15) and a high variance observed with some ++ ewes having high LS (≥ 3) during their reproductive career (Figure 2). This may indicate either a second major prolificacy gene segregating within the Tunisian D'man population or a polygenic effect. In order to test the first hypothesis at the molecular level, we have checked whether mutations in other

known major prolificacy genes (i.e. *FecX*, *FecG* and *FecB*) were involved in the variability of D'man
154 LS. For this purpose, exons 1 and 2 of *BMP15 (FecX)* and *GDF9 (FecG)*, and exon 7 of *BMPR1B (FecB)* genes were sequenced. Apart the already known polymorphisms ΔCTT/B1 in *BMP15* and G3,
156 G4, G5 and G6 in *GDF9* (Hanrahan et al., 2004; Demars et al., 2013, Table 5) we did not identify any other mutations in these genes associated with prolificacy confirming the results from Vacca et
158 al. (2010). Additionally, we also sequenced the exon 7 of *BMPR1B* to genotype for the Booroola mutation ($FecB^B$, OAR6:29382188A>G, NC_019463.1; Mulsant et al., 2001). The $FecB^B$ prolific
160 allele was absent, but we have observed the recently evidenced SNP (OAR6:29382337G>A) shown to be associated with prolificacy in the Iranian Mehraban breed (Talebi et al., 2018). As shown in the
162 Table 4, even if A/A ewes exhibited a lower mean LS (1.68 ± 0.49, n=6) than G/A (1.97 ± 0.45, n=5) or G/G ewes (2.22 ± 0.19, n=3), no significant association was evidenced with the prolificacy of these
164 D'man ++ ewes. Consequently, we failed to validate the existence of a second major gene segregating in our D'man samples.

166 # 4 DISCUSSION

Based on a first public genomic data extraction from the NextGen project and subsequent molecular
168 genotyping, we have evidenced the segregation of the prolific $FecL^L$ allele in the D'man sheep breed from Moroccan and Tunisian populations. Even if quite imprecise due to the relatively low number
170 of animals studied, we have determined a high frequency (0.65) and a rather strong effect (+0.25 lamb/lambing) of the $FecL^L$ allele in the Tunisian D'man sheep, highlighting the presence of a first
172 major gene involved in the genetic determinism of LS variability within this breed known for its high prolificacy. The $FecL^L$ mutation was firstly discovered in Lacaune breed (Drouilhet et al., 2013) and
174 more recently in Noire du Velay (Chantepie et al., 2018), thus raising the question of the origin of this mutation. It is more likely that the $FecL^L$ mutation has an ancestral origin which occurred before the
176 establishment of Lacaune, Noire du Velay and D'man breeds. Previous studies suggested that modern breeds (those with improved traits) were first selected in South-West Asia and then spread into
178 Europe, Africa and the rest of Asia (Chessa et al., 2009). The search and detection of the $FecL^L$

mutation in other ovine breeds worldwide could give more accurate information about its origin. As a first element, other Moroccan sheep breeds (as Beni Guil, Sardi and undetermined local populations) are carriers of the *FecL*^{L} allele as evidenced in the NextGen genomics data (supplementary Table S1), and will require further investigations.

We have observed a large difference in the *FecL*^{L} frequency in the various D'man flocks studied that may result from different selection strategies used by the breeders in these different types of flock. Indeed, the low proportion in ++ individuals found in the commercial RM1 flock is more likely due to an indirect counter-selection of ++ individual carriers through a systematic selection of lambs born as triplets which are most likely born from either *L*+ or *LL* parents (Figure 2), as explained by the breeder itself. This breeding strategy resulted in 94 % of individuals possessing either *LL* or *L*+ genotype (Table 3). Similar proportions of *LL* ewes were found in the RM3 indicating that breeders of the small familiar flocks most probably select for prolific ewes using the same strategy. This decades-old practice used by some D'man breeders could also explain the gap with the only 0.14 allele frequency of *FecL*^{L} observed in the French Lacaune breed (vs. 0.65 in Tunisian D'man) while it is selected on prolificacy since the 80s but with the notable specific exclusion of very prolific animals between 1996 and 2010 (Martin et al., 2014). Interestingly, when looking at the NextGen data (supplementary Table S1), the Moroccan D'man individuals also exhibit a very high frequency (0.58) of the *FecL*^{L} allele with 12 homozygous carriers among the 30 randomly chosen animals.

The present study highlights the segregation of a single mutation with a major effect on prolificacy in the D'man breed thus bringing an important element of answer to the old debate about its existence. Indeed, the high LS, the high repeatability of ovulation rate and LS observed in D'man ewes (and F1 crosses) were possible indicators of the segregation of a prolificacy major gene. However, these and other estimates were not clearly different from Romanov and its crosses in which the inheritance pattern of LS is polygenic (Bradford et al., 1989; Ricordeau et al., 1990; Boujenane et al., 1991). The difficulties experienced in the previous studies to conclude or not for the presence of a prolificacy major gene in the D'man breed may be due to the relatively high frequency of the *FecL*^{L} prolific allele with 80-90% of carrier animals.

206 From this study, we have established the *FecL*^{L} allele effect on prolificacy of D'man ewe around +0.25 lambs per lambing. This is lower than the +0.4-0.5 lambs per lambing observed in Lacaune
208 (Martin et al., 2014) or in Noire du Velay breeds (Chantepie et al., 2018). The model used in the present study did not consider several factors that were shown to affect LS such as lambing rank, age
210 at first lambing, interval since last lambing and season of lambing. The failure to control for these factors may cause allelic effects to become confounded with variation at these factors, leading to
212 underestimation of the true allelic effects. In its existing state, the Tunisian sheep recording system lacks complete information, in particular for the minority breeds among which the D'man breed.
214 Anyway, we have demonstrated that the *FecL*^{L} prolific mutation firstly discovered in the Lacaune sheep has also an effect on the D'man's genetic background. The present results do not provide direct
216 information about the mechanism of action of the *FecL*^{L} mutation, but it seems robust across the different genetic backgrounds in which it expresses itself to control OR and LS (Lacaune, Noire du
218 Velay, D'man). The precise mechanism of action of the intronic *FecL*^{L} on the *B4GALNT2* gene regulation and over expression within the granulosa cells is still unknown. Several hypotheses such
220 as the impairment of transcription factor or miRNA binding, and mRNA alternative splicing were tested *in vitro* or *in silico* but without any success (Drouilhet et al., 2013). The use of high-throughput
222 sequencing technologies dedicated to chromatin accessibility and detection of all kind of RNA (small or long non-coding RNA, mRNA) in the presence or absence of the mutation could contribute to
224 understand the mechanism in the future. Interestingly and independently of the presence of the *FecL*^{L} mutation, some other polymorphisms of the *B4GALNT2* gene have been recently showed to be
226 associated with LS variability in Small Tail Han sheep (Guo et *al.*, 2018), reinforcing the important role of this gene in the control of sheep prolificacy.
228 From a more practical point of view, the discovery of the segregation of the *FecL*^{L} mutation in the D'man breed can help to establish planned genotype-based mating allowing both higher profit for the
230 breeders and an optimal management of prolificacy in D'man sheep populations.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

SBJ, NL and SF designed the study. SBJ, JR and SF performed the blood sampling. FW, JS and SF performed the genotyping. SBJ and SF performed the data analyses and wrote the manuscript.

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Table 1. Ovine prolific alleles

Gene/Locus/ Prolific allele	Chr	Position (v3.1)	Reference allele	Variant allele	Variant ID	NextGen Pop./ Allele freq.
B4GALNT2						
FecL/L	11	36938224	T	A	rs588626728	MOOA T: 0.82; A: 0.18
BMP15	X	50971020	C	A	NA	ND
FecX/B						
FecX/O	X	50971111	T	G	NA	ND
FecX/L	X	50971158	C	T	NA	ND
FecX/Gr	X	50971170	G	A	NA	ND
FecX/I	X	50971224	T	A	rs398521635	ND
FecX/H	X	50971249	G	A	rs413916687	ND
FecX/G	X	50971402	G	A	rs425019156	ND
FecX/R	X	50971643	TGGGTCCAGAAAAGCCCA	T	rs421419167	ND
FecX/Bar	X	50977117	GCTACACTAGC	GGCTACACA	NA	ND
BMPR1B						
FecB/B	6	29382188	T	C	rs418841713	ND
GDF9						
FecG/T	5	41841117	T	G	NA	ND
FecG/H	5	41841212	G	A	NA	ND
FecG/WN	5	41841285	C	T	rs403536877	ND
FecG/E	5	41841362	A	C	rs1092755620	ND
FecG/V	5	41841453	G	A	NA	ND

246 Chr: Chromosome; MOOA : MOroccan Ovis Aries; NA: not available; ND: not detected.

Table 2. PCR primer sequences

Gene (Chr. NCBI RefSeq)	Amplified region	[a]Primer sequence (5'3')	Application
B4GALNT2 (NC_019468.1)	SNP intron 7	Fwd: TCTCAAGGCATTTTGGAGGA Rev: TATGCATGGCATGTGATAGG	KAPA PCR
		Fwd allele 1: GCAAGAAGCTGCGTGTGT Fwd allele 2: GCAAGAAGCTGCGTGTGA Rev: TATGCATGGCATGTGATAGG	KASP genotyping
GDF9 (NC_019462.1)	exon 1	Fwd: GAAGACTGGTATGGGGAAATG Rev: CCAATCTGCTCCTACACACCT	Sanger sequencing
	exon 2	Fwd: TGGCATTACTGTTGGATTGTTTT Rev: GCTCCTCCTTACACAACACACAG	Sanger sequencing
BMP15 (NC_019484.1)	exon 1	Fwd: CACAAAGGATAGGGCAAGGA Rev: ACTTTTCTTCCCCATTTTCTCCC	Sanger sequencing
	exon 2	Fwd: CGCTTTGCTCTTGTTCCCTC Rev: GGCAATCATACCCTCATACTCC	Sanger sequencing
BMPR1B (NC_019463.1)	exon 7	Fwd: AGTGTGTTGGGGGATTTA Rev: AAAGAGAGGAAAGCTAGGAA	Sanger sequencing

[a] Fwd and Rev: Forward and Reverse primers

Table 3 : Genotype frequencies for the 183 genotyped D'man individuals. The numbers in brackets refer to the genotype frequencies in rams.

Genotype	Number	Overall frequency	Frequency per rearing mode		
			RM1	RM2	RM3
++	23	12.5 %	5.5% (6%)	41% (80%)	11% (30%)
L+	81	44.3%	46% (44%)	41% (20%)	42% (70%)
LL	79	43.2%	48.5% (50%)	18% (0%)	47% (0%)

Table 4. Effects of *FecL* genotypes on litter size in the D'man population (mean ± sd and 95 % confidence interval (CI)). Results are averaged over the levels of the three rearing modes (RM1, RM2 and RM3).

Genotype	Number	Mean ±sd	95 % CI
++	15	1.93[a]± 0.18	[1.57-2.29]
L+	66	2.23[a]± 0.09	[2.05-2.41]
LL	71	2.47[b]± 0.09	[2.29-2.66]

[a, b] means with different superscripts differ significantly (P <0.05).

Table 5. Polymorphisms detected in *FecL* +/+ ewes at various prolificacy major gene loci and association with litter size (LS).

Gene (Chr. NCBI RefSeq)	Polymorphism	Genotype	n=	LS (mean ± sd)
FecX/BMP15	X:50977457delinsCTT (B1) [a]	Ins/Ins	7	1.69 ± 0.45
(NC_019484.1)		Ins/Del	6	2.05 ± 0.45
		Del/Del	2	2.00 ± 0.47
FecG/GDF9	5:41841919A>G (G3) [a]	A/A	2	2.17 ± 0.24
(NC_019462.1)		A/G	8	1.82 ± 0.58
		G/G	5	1.90 ± 0.32
	5:41841675A>G (G4) [a]	A/G	1	2.33 ± ND
		G/G	14	1.86 ± 0.46
	5:41841418A>G (G5) [a]	A/A	11	1.89 ± 0.38
		A/G	3	1.89 ± 0.84
		G/G	1	2.00 ± ND
	5:41841402G>A (G6) [a]	G/G	11	1.89 ± 0.38
		G/A	3	1.89 ± 0.84
		A/A	1	2.00 ± ND
FecB/BMPR1B	6: 29382337G>A [a]	G/G	4	2.22 ± 0.19
(NC_019463.1)		G/A	5	1.97 ± 0.45
		A/A	6	1.68 ± 0.49

[a] no significant association between genotypes and LS was detected by one-way ANOVA

FIGURE LEGENDS

Figure 1. Proportions of the number of litter size records for D'man ewes sampled for the study (n = 152).

Figure 2. Proportion of litter size depending on the *FecL* genotypes.

The histograms show the distribution (percentage of the whole dataset, n=488 lambing records) of the lambing mode, simple (LS=1), twin (LS=2), triplet (LS=3) and quadruplet and more (LS=4+) depending on the 3 genotypes at the *FecL* locus (++, L+ and LL).