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Breeding scheme for the introgression of prolificacy gene into Barbarine sheep

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Contents

1. Background

Sheep husbandry is a main pillar of the red meat value chain in Tunisia. The Barbarine fat tailed sheep breed is the most important one in number, adaptation, biodiversity and history in the country. Physically, the Barbarine has an open face and its extremeties (head, ears, legs) may be black, brown or red. Several breeding programs are being implemented to sustain genetic improvement of meat producing sheep breeds in Tunisia and the focus has been on improving growth traits. The litter size (LS) or prolificacy is of high economic importance. Considering this trait, most of the sheep breeds in Tunisia are low prolific. Thus, a prolificacy-based selection program was implemented since 1979 by the Tunisian National Institute for Agronomic Research (INRAT) in the experimental center of Oueslatia (Kairouan) by screening prolific ewes among the fat tailed Barbarine breed which represents over 60% of the national sheep population. To take advantage of this "W-INRAT" prolific line, INRA Tunisia and ICARDA joined efforts for a sustainable partnership in applying science and technology to help farmers improve their livelihood under harsh conditions. Thus, we conducted within the framework of CGIAR Research Program on Livestock-Genetics Flagship, our project entitled "Research activities on introgression of prolificacy gene into Barbarine sheep" to improve reproductive traits in meat sheep flocks. This project aimed at introgressing the prolificacy mutation into commercial flocks of the Barbarine breed through the establishment of a breeding scheme backed by genomic screening of the mutation. In addition to INRAT and ICARDA expertise, the project was backed up by the “Livestock Development and Pasture Improvement National Program” (OEP, Ministry of Agriculture) to support cervical artificial insemination in conventional flocks.

The specific project objectives are:

I. Improving prolificacy in conventional flocks of the Barbarine breed for mid and long term use by dissemination of the prolificacy mutation selected in a single flock "W" at the national level,
II. Creation of novel improved prolific rams’ centers in the North-West (OTD-Kodiat) and in the Center of the country (OEP-Saouef),

III. Determine the optimal strategy (combination of matings) which maximizes the economic gain across the genotype effects to be implemented at the nucleus and commercial levels.

2. Animals and flocks' management

The project was implemented in two public commercial farms:

- OEP-Saouef: The sylvo-agro-pastoral pilot farm (Office de l'Elevage et des Pâturage, OEP) under semi-arid conditions (330 mm average annual rainfall) located in Saouef region in the governorate of Zaghouan.
- OTD-Kodiat: The "Kodiat" farm belonging to Office des Terres Domaniales, OTD in the region of Bousalem (governorate of Jendouba) located in the North west of Tunisia.

Four flocks of conventional Barbarine ewes from the black-headed ecotype were considered in this project. In OEP-Saouef farm: a total of 251 conventional ewes were clustered into three groups and inseminated. The criteria for clustering were based on body condition, prolificacy and lambing precocity in previous years. In OTD-Kodiat farm, a total of 100 conventional ewes were inseminated.

The ewes of both farms were maintained on natural rangeland throughout and received, in addition, a daily concentrate supplement (400g/ewe/day). Ewes in both flocks remained in isolation from rams for at least two months before the date of insemination. Animals had access to water at all times. The rams were allowed to graze available vegetation and they were daily supplemented with 0.7 kg/ram of barley-based concentrate.

Photo1. Conventional black-headed ewes in Saouef farm

In each of the 2 previous farms, the following data was collected: number of sheep kept; number of artificially inseminated ewes per flock; number of progeny born and weaned after AI per flock; method of collecting and qualifying semen to use; level of dilution, kind of extender and the size of inseminating dose; method of estrus synchronization; method of insemination; control of pregnancy and use of ram natural service at return oestrus after insemination.

3. Artificial Insemination (AI)

3.1. Rams training for semen collection

A total of seven prolific Barbarine rams (carrier of mutation) were moved from Oueslatia (INRAT) station to Saouef farm to be used for semen collection and artificial insemination. Rams were trained to for only 2 weeks to ejaculate in artificial vagina using oestrous-induced ewes. An experienced technician evaluated their performances and only four rams (4073, 8283, 4034 and 4032) were selected to be later used in artificial insemination.

3.2. Reproduction calendar

The planning of AI steps held in the eight groups of the four conventional flocks are shown in table (1). In March-April, intravaginal pessaries were inserted in 351 ewes and left in-situ for 14 days. At sponge withdrawal, ewes in the first treatment group received 400 I.U. of equine chorionic gonadotropin and remained isolated from rams. Ewes were inseminated with fresh semen approximately 55 hours after sponge removal.

Table 1. Calendar of flock's reproduction management

Farm	Flock	Group	#Ewes	Melovine implant	Sponges insertion	Sponges removal	AI
OEP-Saouef	7	1	50	28/02	26/03	9/04	11/04
	7	2	50	28/02	2/04	16/04	18/04
	7	3	16	28/02	15/04	29/04	01/05
	9	1	50	28/02	25/03	8/04	10/04
	9	2	50	28/02	02/04	16/04	17//04
	9	3	35	28/02	15/04	29/04	01/05
OTD	4	-	50	-	29/04	13/05	15/05
	3	-	50	-	2/05	16/05	18/05

3.3. Melovine implantation

Prior to artificial insemination, starting end of February, and in order to reduce the intensity of seasonal anoestrus, the "W" rams and only conventional ewes from (OEP-Saouef) received melatonin implants. Melatonin was administered in the form of a subcutaneous implant, Melovinee® (CEVA-Animal Health, Tunis, Tunisia), near the base of the ear using a Melovinee applicator gun (photo 2).Throughout the experimental period, rams were permanently kept outdoors under conditions of natural day length and at ambient temperature.

Photo 2. Application of Melovine implants

3.4. Synchronization with progestogen-impregnated sponges

After 45 days from Melovinee implantation, all ewes were synchronized using intravaginal pessaries impregnated with 30 mg Acétate de fluorogestone (Syncro-Part®CEVA – Animal Health, Tunis, Tunisia). Sponges were inserted and left in-situ for 14 days.

Photo 3. Intravaginal sponge insertion and removal

At sponge withdrawal, conventional ewes were immediately injected with PMSG (Syncro-Part PMSG-6000UI) at a dose 400 I.U./female equivalent to a volume of 2 ml and were continuously checked for oestrus by experienced technicians. Ewes detected in oestrus

within the first 36 hours or later were respectively inseminated with fresh semen at approximately 55±1 hour using fine pellets containing 0.25 ml of diluted fresh semen containing $400x10^6$ spz.

Photo 4. Injection of 400 UI PMSG immediately after removing sponges

3.5. Semen collection and evaluation

Semen was collected by artificial vagina from four adult rams with proven libido, previously trained for semen collection. At each occasion, the rams were put individually in the collection room in presence of a teaser female that was previously induced into estrus. Ejaculates are recovered in graduated glass tubes (4 ml). This allowed direct determination of the ejaculate volume without taking into account the frothy part on the top. Semen collection was performed by the same operator and ejaculates were immediately kept in a water bath at 35°C. Each ram was used until three times /day at constant intervals. In our case, the semen from different ejaculates was at random attributed to Barbarine ewes at a mean ratio of 1 ram for every 86 ewes.

Photo 5. Different steps of semen collection, evaluation and insemination

Immediately after collection, the evaluation of semen quality is based on macroscopic observation: Volume (graduated tube), color and aspect and microscopic measures: concentration, individual and massal motility of spz. The mass activity (wave motion or motility score) in undiluted semen was assessed by examining a drop of semen under a warm stage using a phase contrast microscope at ×100 magnification (score, 0-5).The fresh semen is diluted with a skimmed-milk diluent at 32-35 °C and put into fine pellets of a 0.25ml volume. Four microliters of fresh semen were diluted in 3996 µl of physiological saline solution. The concentration was determined using a spectrophotometer that is calibrated to measure sheep sperm concentration at 550 nm (Accucell®, IMV, Paris, France). We only used ejaculates presenting a minimum concentration of $3x10^9$spz/ml, measured by spectrophotometry at 540 nm (Accucell®, Instruments de MédecineVétérinaire, IMV Paris, France) and a progressive motility of 3 out of a scale of 5 and motility of 30% and percentage of living spz of 60%. The cervical insemination was carried out by the same experienced technician using 0.25 ml of diluted semen containing in average 400 x 10^6 sperm.

4. Overview of AI results

The conception rate to AI is calculated as the proportion of lambing ewes from inseminated ones. In our case the conception rate to AI in different flocks ranged between 32 to 41% (Figure 1). Although the flock 7 in OEP-Saouef farm recorded the highest conception rate to AI (41%), it presented the lowest rate of prolificacy (108.9 %, Figure 2). For all the inseminated ewes, we observed 83.5 % single births and 16.5% twin births (Figure 3). The sex of offspring animals presents relatively equal proportions: 46.5 % males vs 53.5 % females. We also found that the highest proportion of males born was observed in flock 9 in OEP-Saouef farm and the highest male ratio (the proportion of males from total number of offspring born in the flock) was shown in flock 3 in OTD-Kodiat farm (Figure 4). This indicator is important because we are interested to obtain the maximum males in F1 generation in order to better disseminate mutations of gene prolificacy in future generations.

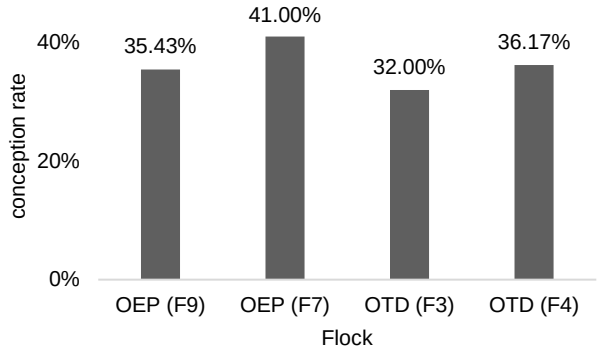

Figure1. Conception rate to AI in each flock

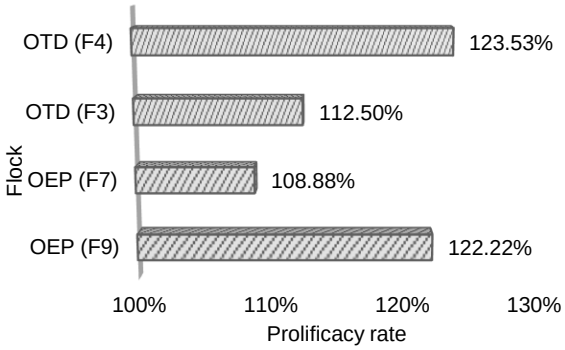

Figure 2. Litter size of artificially inseminated Barbarine ewes synchronized with progestogen-impregnated sponges and equine chorionic gonadotropin

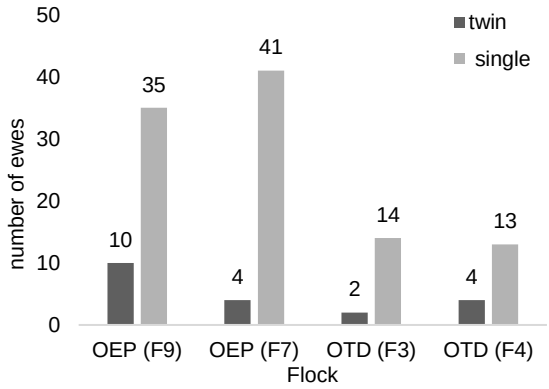

Figure 3. Number of ewes giving birth to singles or twins in each farm

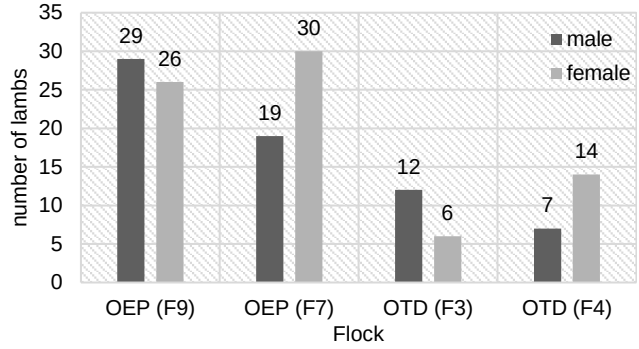

Figure 4. Distribution by sex of the offspring

5. The genotyping of BMP15 and GDF9 gene prolificacy in conventional ewes

Details about AI of a total of 118 inseminated ewes were recorded: AI date, ram ID, semen quality evaluation, lambing date, litter size, sex of lambs, offspring ID and date of natural mating for ewes returning to service. Blood samples from both recipient ewes and offspring were randomly collected and genotyped for mutations in BMP15 and in GDF9 genes using AS-PCR and PCR-RFLP methods, respectively.

5.1. Polymorphism genotyping of BMP15 gene using Allele-specific PCR

A genotyping test by allele specific PCR amplification was established for the detection of the *FecX*^{Bar} mutation in conventional Barbarine flocks. For each individual, a fragment of 445 (or 440) bp from BMP15 exon 1 is amplified by two independent PCR using the same forward primer 5′-TTCCTTGCCCTATCCTTTGTG-3′ and one of the two allele specific reverse primer, 5′-GAGGCCTTGCTACACTAGCC-3′for the FecX^{+} wild-type allele and 5′-TGAGAGGCCTTGGCTACACA-3′ for the FecX^{Bar} mutated allele. After 35 cycles of amplification (94 °C/30s, 60 °C/60s, 72 °C/60s), the PCR products were analyzed on 1% agarose gel. In silico PCR, primers amplified a fragment of 442 bp size ranging from position 50986400pb to 50986841pb in the exon 1 of *BMP15* gene on chromosome:

TTCCTTGCCCTATCCTTTGTGgtagtggagcctggatgctgttacccatgtaaaaggaaaggtttaaagcgttatcctttgggcttttatcagaacatgttgctgaacaccaagcttttcaagatggtcctcctgagcatccttagaatccttctttggggactggtgcttttttatggaacatagggtccaaatgacacaggtagggcagccctctattgcccacctgcctgaggcccctaccttgcccctgattcaggagctgctagaagaagcccctggcaagcagcagaggaagccgcgggtcttagggcatcccttacggtatatgctggagctgtaccagcgttcagctgacgcaagtggacaccctagggaaaaccgcaccattggggccaccatggtgaggctggtgaggccgct**GGCTAGTGTAGCAAGGCCTC**

Table 2. Allele specific PCR primers of ovine *BMP15* gene

BMP15 primer	Primer sequence (5′→3′)	Nomenclature
Long Forward	TTCCTTGCCCTATCCTTTGTG	F+
Wild allele	GAGGCCTTGCTACACTAGCC	R+
Mutated allele	TGAGAGGCCTTGGCTACACA	R-

Table 3. Results of *BMP15* genotyping in conventional ewes in OEP farm: flocks n°7 and n°9

Flock	ADN N°	PCR	Genotype
7	7P1	FR+	x/x
7	7P2	FR+	x/x
7	7P3	FR+	x/x
7	7P4	FR+	x/x
7	7P5	FR+	x/x
7	7P7	FR+/FR-	x/bar
7	7P8	FR+	x/x
7	7P9	FR+ / FR-	x/bar
7	7P10	FR-	bar/bar
7	7P13	FR+	x/x
7	7P14	FR+	x/x
7	7P15	FR+	x/x
7	7P16	FR+	x/x
7	7P17	FR+	x/x
7	7P18	FR+	x/x
7	7P19	FR+	x/x
7	7P23	FR-	bar/bar
7	7P28	FR+	x/x
7	7P29	FR+	x/x
7	7P30	FR-	bar/bar
7	7P31	FR+	x/x
7	7P32	FR+	x/x
7	7P33	FR+	x/x
7	7P34	FR+	x/x

Flock	ADN N°	PCR	Genotype
9	9P2	FR+	x/x
9	9P3	FR-	bar/bar
9	9P5	FR+	x/x
9	9P7	FR+/FR-	x/bar
9	9P9	FR+	x/x
9	9P12	FR+	x/x
9	9P13	FR+	x/x
9	9P14	FR+	x/x
9	9P15	FR+	x/x
9	9P16	FR+	x/x
9	9P17	FR+	x/x
9	9P18	FR+	x/x
9	9P19	FR+	x/x
9	9P20	FR+	x/x
9	9P21	FR+	x/x
9	9P22	FR+	x/x
9	9P23	FR+	x/x

Heterozygous ewes were positive for the two PCR while homozygous ewes were positive only for one PCR depending on the carried allele. The rams were expected to be positive only for one of the two PCR's.

The genotyping of *BMP15* gene using AS-PCR technique showed that, among the genotyped conventional ewes, we detected the same mutation found in prolific "W" line in nine samples from OEP flocks with 4 heterozygous carriers (x/bar). and 5 homozygous mutated (bar/bar). This was explained that the mutation was conserved since 2000 when "W" breeding rams' carriers of the mutation were used for mating the ewes from OEP farm in the framework of a federative project between INRAT, OEP and IRESA (Bedhiaf, Ben Hamouda, Rekik, Ben Sassi, 2000).

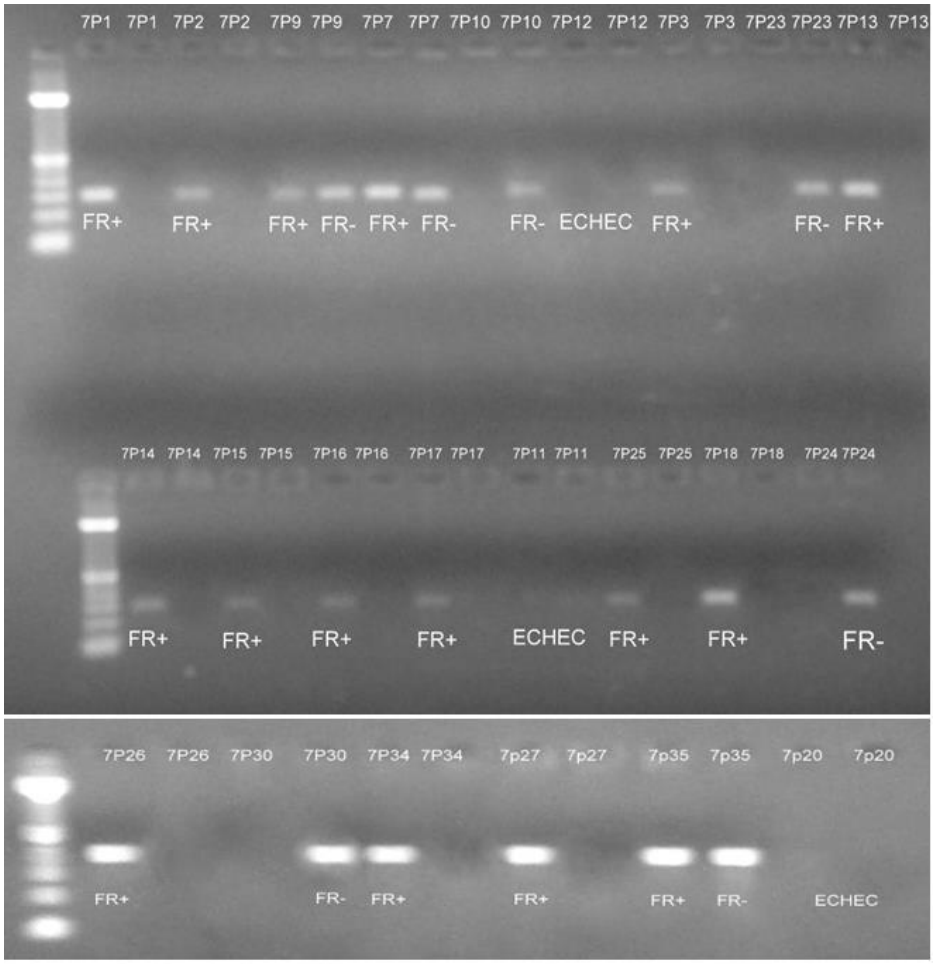

Figure 5. Agarose gel migration of AS-PCR amplification at the *FecX* ^{Bar} locus in flock n°9

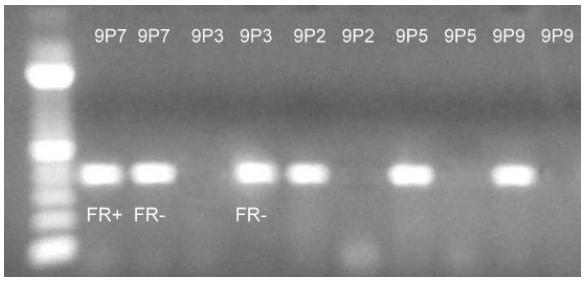

Figure 6. Agarose gel migration of AS-PCR amplification at the *FecX* ^{Bar} locus in flock n°9 *FecX* ^{Bar} *and FecX* $^{+}$*allele specific PCR amplification of genomic DNA from Barbarine non-carrier ewes (x/x), heterozygous (x/bar) and homozygous carrier (bar/bar) of the FecX* ^{Bar}*allele. Amplified bands were resolved on 1% agarose gel (Figure Gel migration).*

5.2. Polymorphism genotyping in GDF9 gene using PCR-RFLP

The FecG allele genotyping was performed through Restriction Fragment Length Polymorphism (RFLP) using the genomic DNA from 23 conventional females. Restriction enzyme digestion by DdeI (Takara Bio) of each specific PCR product was performed for one hour at 37 °C. One pair of primer (Forward 5′-ATGGATGATGTTCTGCACCATGGTGTGAACCTGA-3′ and Reverse 5′-CTTTAGTCAGCTGAAGTGGGACAAC3′) was designed to amplify 139 bp PCR product of sheep GDF9 gene. The 50 µL volume containing: 50 to 150 ng genomic DNA(~ 6 ul), 3µl for each primer (10µM) (carthagenomicscompagny) , 2.5µl of MgCl2 (25 mM) , 5µl of dNTPs (dATP, dTTP, dCTP, dGTP) 2mM, 0.5µl DMSO, 5µl ofHOT FIREPol® 10x Buffer B1 containing (Mg2+ and detergent free) Tris-HCl and (NH4)2SO and the final volume was adjusted to 50µl using waterDnaseRnase free.The cycling protocol was 4 min and 50 secondes at 94°C , 35 cycles of 94°C for 30 s, 60°C annealing for 60 s, 72°C for 1 min, with a final extension at 72°C for 10 min.

The PCR products were analyzed on 1% agarose gel. In silico PCR, primers amplified a fragment of 139bp size ranging from position 41768261pb to 41768399pb in GDF9 on chromosome5:ATGGATGATGTTCTGCACCATGGTGTGAACCggagagccataccgatgtccgaccgccctgggacagtccccctttacagtatcgagggttgtatttgtgtggggccacaatccaGTTGTCCCACTTCAGCTGACTAAAG

Table 4. PCR-RFLP genotypes of FecG mutation in GDF9 gene in 22 conventional ewes

Flock	Sample	PCR &DdeI enzyme	Genotype
7	7P1	alleles 139pb	Homzygote mutated
7	7P2	alleles 139pb	Homzygote mutated
7	7P3	alleles 139pb	Homzygote mutated
7	7P4	alleles 139pb	Homzygote mutated
7	7P5	bande 139pb	Homzygote mutated
7	7P7	alleles 139 et 30	homozygote wild
7	7P8	alleles 139 et 30	homozygote wild
7	7P9	alleles 139 et 30	homozygote wild
7	7P10	alleles 139 et 30	homozygote wild
7	7P11	alleles 139 et 30	homozygote wild
7	7P12	alleles 139 et 30	homozygote wild
7	7P14	alleles 139 et 30	homozygote wild
7	7P15	alleles 139 et 30	homozygote wild
7	7P19	alleles 139pb et 30	homozygote wild
7	7P31	bande 139	homozygote mutated
9	9P12	allele 139 et 30 bp	homozygote wild
9	9P14	allele 139 et 30 bp	homozygote wild
9	9P16	allele 139 et 30	homozygote wild
9	9P19	allele 139 et 30	homozygote wild
9	9P20	allele 139 et 30	homozygote wild
9	9P21	allele 139 et 30	homozygote wild
9	9P25	allele 139 et 30	homozygote wild
9	9P26	allele 139 et 30	homozygote wild

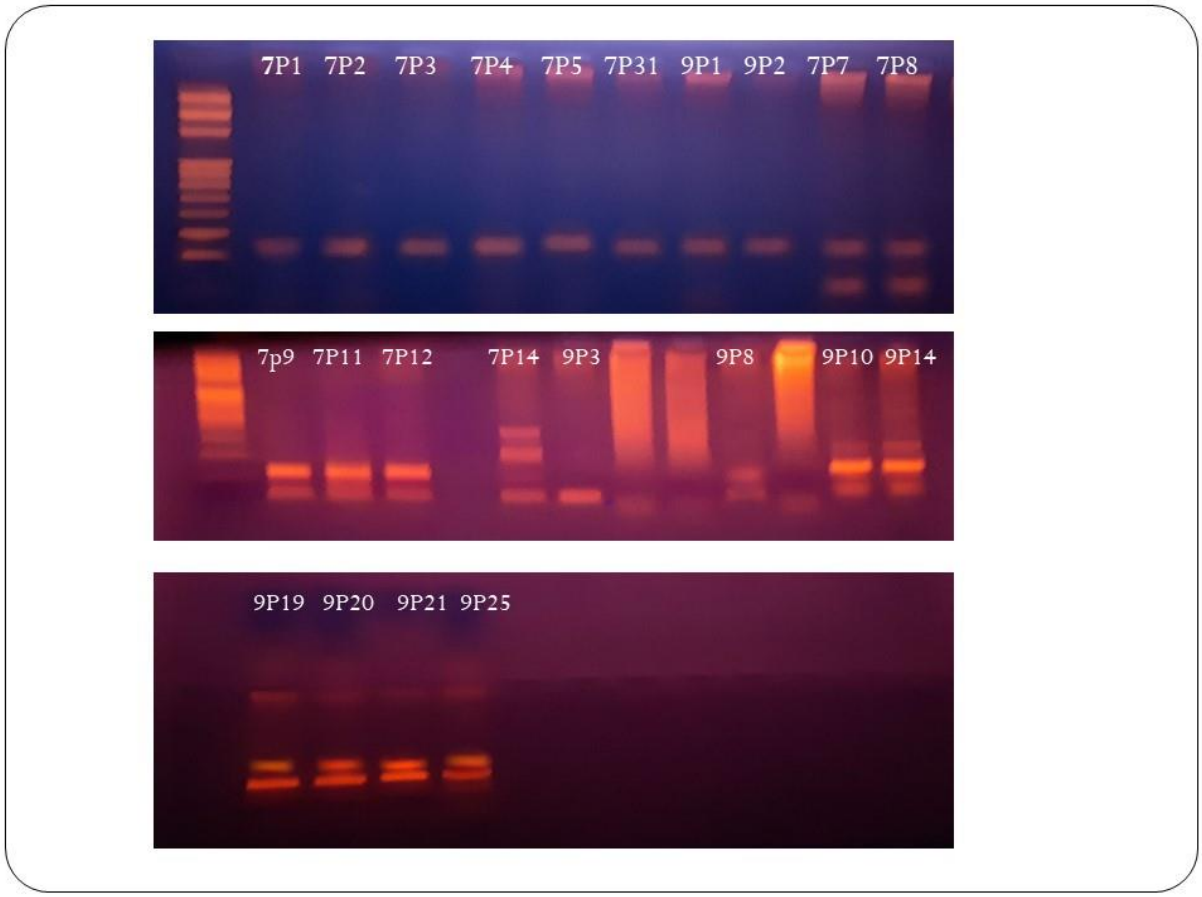

Figure 7. The PCR-RFLP genotypes of GDF9 (FecG) gene in conventional Barbarine ewes

In conclusion, these findings provide the first evidence that mutations in *GDF9* and *BMP15* are associated with the reproductive effects. The increased ovulation rate and the majority of the sterility phenotypes in these animals can be explained by the presence of heterozygous mutations and homozygous mutations, respectively, in these genes. The X-linked effects of the *BMP15* and the autosomal *GDF9* mutations were required to explain the variation seen in "W" flock and in their offspring.

6. Experimental protocol and setting up a breeding scheme

Rams had been progeny tested: Analysis was carried out on DNA from "W" rams involved in the progeny testing program and on test-cross daughters of four of these rams. The AI applied in nucleus flocks was performed by using fresh semen of the 4 prolific rams, carriers of FecX mutation with 350 conventional ewes belonging to four flocks in two farms (OEP and OTD) (Figure 8).

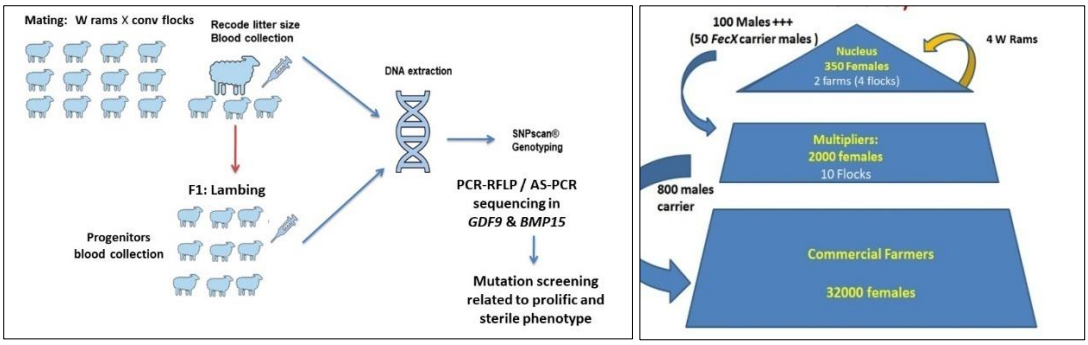

Figure 8. Schematic presentation of the experimental protocol and breeding scheme