RESEARCH COLLABORATION AGREEMENT

BETWEEN

INTERNATIONAL CENTER FOR AGRICULTURAL RESEARCH IN THE DRY AREAS

AND

INSTITUT NATIONAL DE RECHERCHE AGRONOMIQUE DE TUNISIE

WITH THE COLLABORATION OF

OFFICE DE L'ELEVAGE ET DES PATURAGES

&

INSTITUTION DE LA RECHERCHE ET DE L'ENSEIGNEMENT SUPERIEUR AGRICOLES

FOR CONDUCTING

RESEARCH ACTIVITIES ON INTROGRESSION OF PROLIFICACY GENE INTO BARBARINE SHEEP

WITHIN

CGIAR RESEARCH PROGRAM ON LIVESTOCK – GENETICS FLAGSHIP

RESEARCH ACTIVITIES ON INTROGRESSION OF PROLIFICACY GENE

INTO BARBARINE SHEEP

Agreement Number:No.:200081

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Summary

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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 points may be black, brown or red. Several breeding programs are being implemented to sustain genetic improvement of sheep meat production. 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 sheep 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 aims at successfully completing introgression of prolificacy mutation in conventional non-prolific Barbarine flocks in the framework of a breeding scheme. In addition to INRAT and ICARDA expertise, the project is backed up by the "Livestock Development and Pasture Improvement National Program" (OEP, Ministry of Agriculture) to support cervical artificial insemination in conventional flocks in saving the genetic integrity of the Barbarine breed and improve its productivity within a market oriented framework.

2. Conceptual framework and methodology

The document reports the achievements and on-going activities within the framework of this project conducted by the INRAT-OEP team with the collaboration of ICARDA in the area of Ouesslatia and Saouef located in the districts of Kairouan and Zaghouan, respectively. A series of skype meetings between ICARDA and INRAT scientists were held to finalize the protocol and were followed by meetings with OEP staff and group discussions to explain the protocol and select recipient flocks. The main activities are:

- Selection of the recipient Barbarine flocks;
- The AI planning: In order to guarantee an appropriate introgression of prolificacy mutation identified in W-INRAT rams, a cervical artificial insemination will be used according to OEP standard protocol;
- The adequate use of fecundity genotyping information along with polygenic background for W-INRAT Barbarine line to achieve the maximum genetic merit;
- Provide a valuable genetic tool to control fecundity in OEP flocks, usable for diffusion program into commercial flocks seeking prolificacy improvement.

3. Flocks' selection

A total number of 400 conventional ewes from two OEP flocks, raised in Saouef station, are used in this study as recipient females; Donors are the prolific rams from W-INRAT flock of Ouesslatia station. Both in INRAT and OEP sheep flocks, a spring mating is practiced using a ram effect and lambs are born during September-November period. In W-INRAT flock, reproduction is managed through seven families where one ram and its replacement were

assigned for each family. For mating, which is practiced in the spring, females born in a given family are systematically moved to another family to limit inbreeding and replacement animals are selected based on their litter size records. Weaning occurs at approximately 3 months of age. Grazing is mainly based on natural pasture. However, concentrate is used as supplement during breeding and late gestation periods. Pedigree and reproductive records of the prolific Barbarine line are collected by the Tunisian National Institute for Agronomic Research (INRAT) and show the prolific "W" strain with LS of 1.61 (table 1) and a fertility rate of 89%. Traits considered were: litter size at birth (LSB), litter size at weaning (LSW), litter weight at birth (LWB) and litter weight at weaning (LWW) (table2). The LS in conventional two flocks (OEP) of the same breed is of 1.13.

Table 1. Description of the W-INRAT flock data set

Recording period	1990-2017
Number of records	2726
Number of sires	195
Number of dams	441
Average age of ewes	2.7
Average number of lambs born per ewe lambing	1.61
Lambs born per ewe lambing (%)	
Single	47.9
Twin	45.8
Triplet	5.9
Quadruplet	0.4

Table 2. Descriptive statistics for litter size (LS), birth weight (BW), weight at 90 days (W90), and average daily gain between 10 and 30 days (ADG13).

Trait	**N**	**Mean**	**SD**	**CV(%)**
LS	2725	1.61	0.62	40.00
BW	3804	2.77	0.55	19.99
W90	3066	14.81	3.87	26.13
ADG13	3090	135.10	54.38	40.25

4. BMP15 and GDF9 sequence analysis

Interestingly, numerous studies in sheep identified point mutations in genes of the bone morphogenetic protein (BMP) family of growth factors associated to increased prolificacy but also to sterility resembling the phenotypes observed in the Barbarine "W" flock. Indeed, up to now, 7 different mutations were evidenced in the bone morphogenetic protein 15 (*BMP15*) gene and 3 mutations in the growth and differentiation factor 9 (*GDF9*) gene responsible for this prolific and sterile double phenotype. The ovine *BMP15* gene is carried by the X chromosome and is also known as *FecX* (Fecundity X gene). The *FecX/BMP15* mutated alleles identified in Barbarine breed was $FecX^{Bar}$. Concerning the *GDF9* or *FecG* gene on the ovine chromosome 5, one mutated allele was identified in Cambridge and Belclare sheep and is known as $FecG^{H}/GDF9^{c1184C>T}$. For both *BMP15* and *GDF9*, all these variants affect the open reading frame. They are considered as loss-of-function mutations increasing ovulation rate (OR) and thus prolificacy at the heterozygous state, but sterility by blockage or deep alteration of the ovarian follicular development at the homozygous state. The search for these mutations in *BMP15* and *GDF9* genes was to characterize of what could be a new introgression of fecundity mutations affecting the prolificacy of Barbarine sheep on its progeny productivity. We initiated the genotyping and sequencing of i) "W" Barbarine rams (INRAT), ii) random representative number of recipient Barbarine ewes (OEP) and iii) molecular characterization is also planned for their offspring.

4.1. Polymorphism genotyping

A genotyping by allele specific PCR amplification for the detection of the $FecX^{Bar}$ mutation was performed: 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. Heterozygous ewes were positive for the two PCR while homozygous ewes were positive only for one PCR depending on the carried allele. The *BMP15* gene is located on the X chromosome, thus males were expected to be positive only for one of the two PCR's.

A second genotyping using PCR-RFLP is on-going work for detection of the mutated allele $FecG^{H}/GDF9^{c1184C>T}$ in the *GDF9* or *FecG* gene on the ovine chromosome 5. The

following primer was designed to introduce a point mutation in 139 bp PCR product of GDF-9 gene. Then the wild type products could be cleaved by DdeI (CTTAG) with a 109 bp and 30 bp fragments, the mutation type with $FecG^H$ remained uncleaved.

Gene	Allele	Enzyme	Primers sequence	lenght	Cleaved products
GDF9	$FecG^H$	DdeI	ATGGATGATGTTCTGCACCATGGTGTGAACCTGA CTTTAGTCAGCTGAAGTGGGACAAC	139 pb	139-109-30

4.2. Sampling and DNA extraction

Blood samples were taken from 10 rams which belong to the W-flock of the National Institute of Agronomic Research of Tunisia (INRAT). The flock is raised in the experimental station of Ouesslatia which is located at 200 km of the south west of Tunis (35° 51' N and 9° 35' E) under a semi-arid climate. Sample size is considered representative as long as the flock includes 103 individuals. Isolation of the genomic DNA was carried out by the standard phenol-chloroform extraction method using Proteinase K digestion (Sambrook et *al*., 1989). The isolated gDNA quality were verified on 0.8 % agarose gel containing ethidium bromide then was stocked at -20°C.

4.3. AS-PCR amplification

Polymerase chain reactions (PCR) were conduct in 50µl total reaction volumes, each containing 5µl buffer Taq BIOMATIC reaction, 2.5µl $MgCl_2$ (25mM), 5µl dNTPs (25mM), 3µl of each primer (10µM), 1.2µl Taq Polymerase (5u/50µl), 5µl genomic DNA and H_2O.

The PCR mixtures underwent a heat phase of 94°C for 3 min, followed by 32 cycles each consisting of 30 sec of denaturation at 94 °C, 55 sec of annealing at 60 °C and 1 min of extension at 72 °C and a final elongation 72°C/10 min. A volume of 7 µl of PCR products were analyzed on 2% agarose gel.

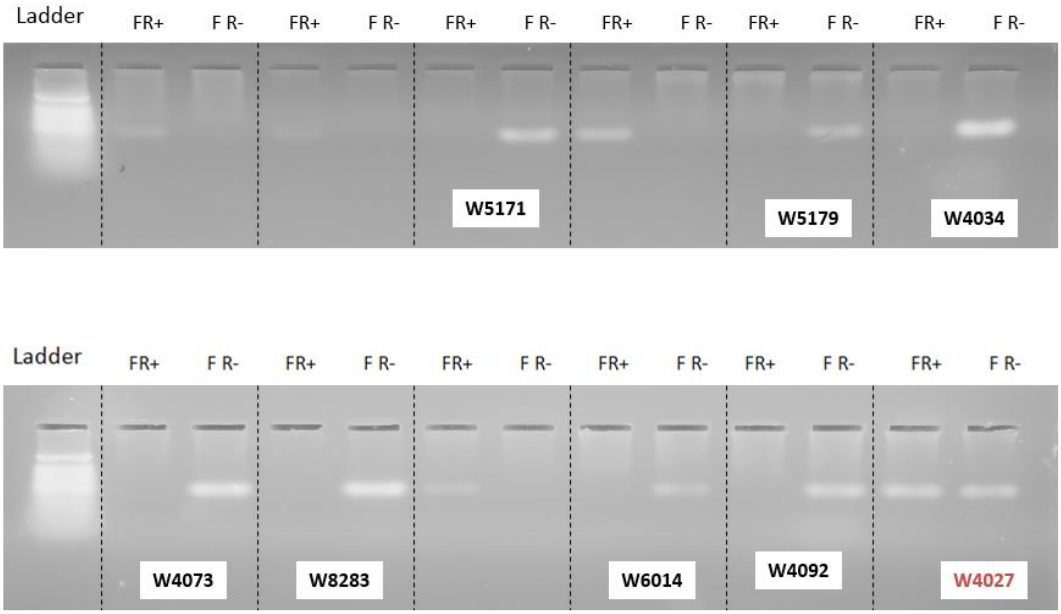


Figure 1. Gel profile of *FecX*^{Bar} mutation in BMP15 exon 1 in Barbarine W rams

Table 3. W-INRAT rams genotypes for *FecX*^{Bar} mutation.

ID_Anim	PCR	Genotype
W5171	FR-	*FecX*^{Bar} /Y
W5179	FR-	*FecX*^{Bar} /Y
W4034	FR-	*FecX*^{Bar} /Y
W4073	FR-	*FecX*^{Bar} /Y
W8283	FR-	*FecX*^{Bar} /Y
W6014	FR-	*FecX*^{Bar} /Y
W4092	FR-	*FecX*^{Bar} /Y

The PCR products were purified using Exonuclease I (Exo I) and the purification kit FastAP^{TM} Thermosensitive, then the purified products will be sequenced using BigDye ® Terminator *v.1.1* Sequencing (Applied Biosystem).

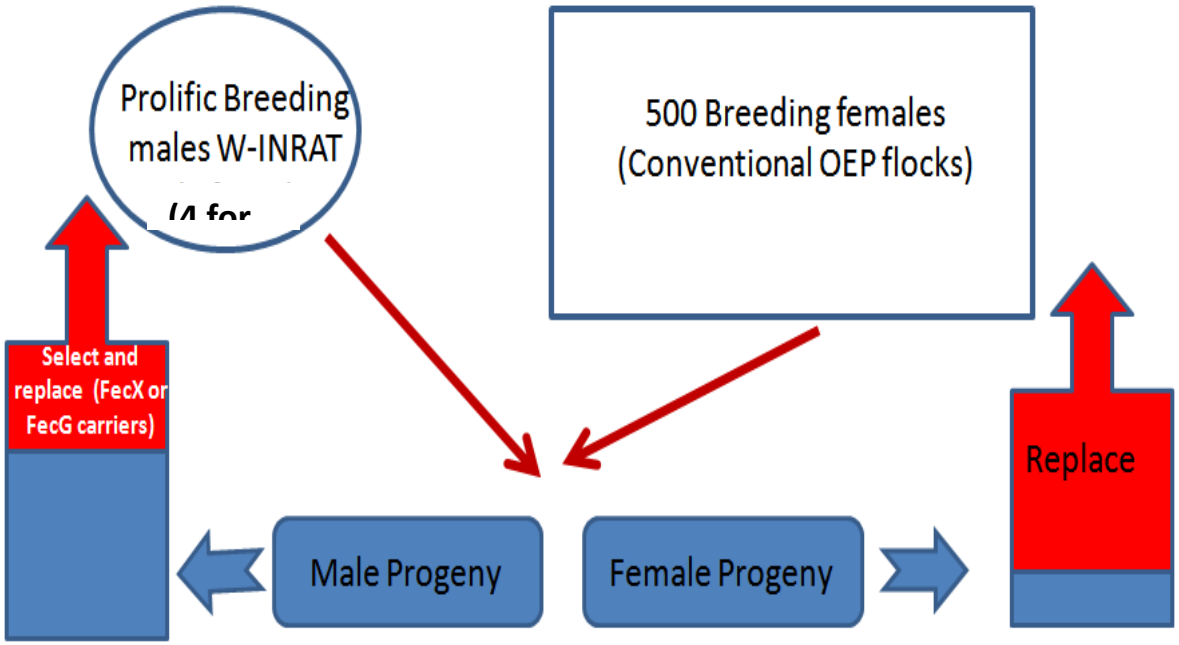
5. Programmed mating

Artificial insemination of non-carrier homozygous recipient ewes (OEP) with carrier rams (W-INRAT). Focus mating within "W" flock: Two flocks belonging to OEP flocks (Saouef station).

- February 2019: Melovine implantation of W-INRAT males
- March 2019: All recipient ewes (OEP) will be checked for non pregnancy and assessed for good body condition and good previous reproduction records.

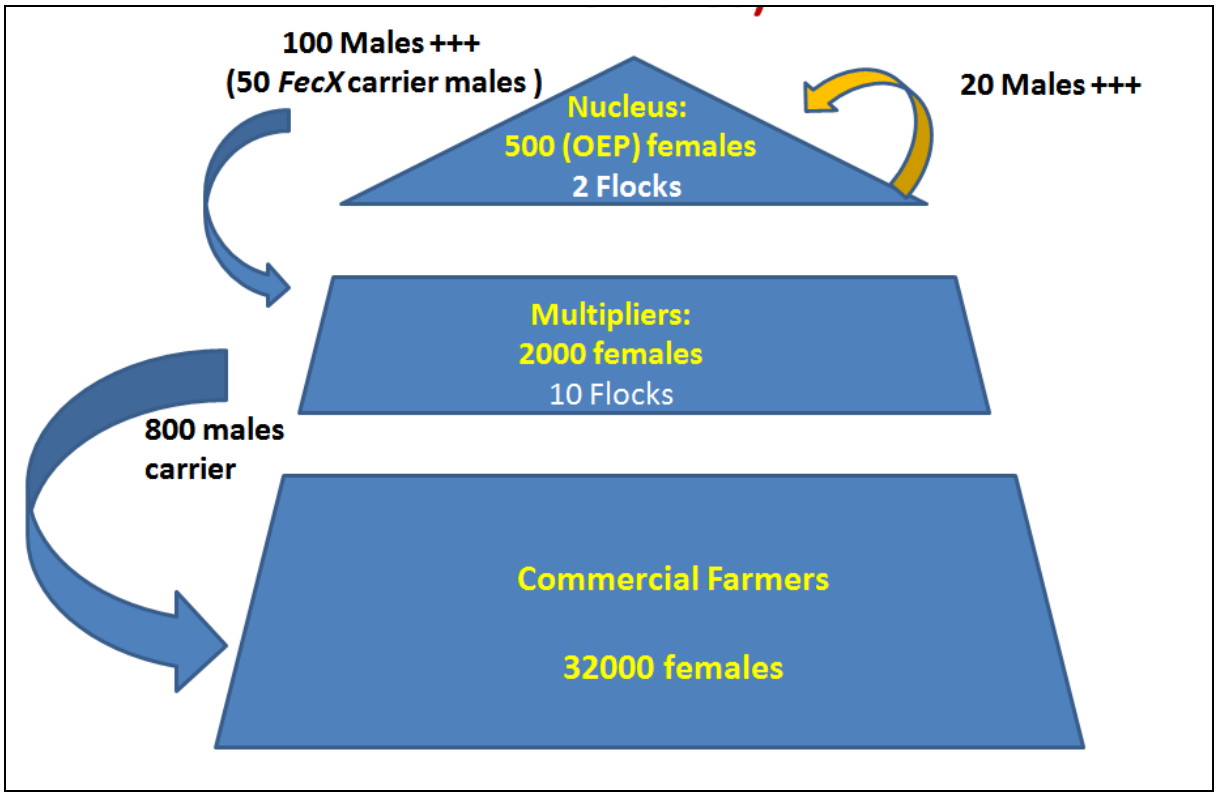
- April 2019: The two flocks will be synchronized using the conventional progestogen-eCG protocol.
    - Fresh semen collected from 4 W- INRAT rams.
    - Cervical AI.

1st step: March-Mai 2019



2nd step: February- April 2020

Nucleus: Using cervical AI for the 500 breeding females (non-carrier of the *FecX* mutation), only *FecX* carrier offspring males will be selected. We will establish a multiplier layer of 2000 conventional females which will be mated to the 50 *FecX* carrier males from the nucleus (800 male offspring, easy to sire 32 000 females).



In the next stage, the activities planned at:

The Nucleus level:

- Performance and pedigree recording at OEP flocks;
- Breeding values evaluation;
- Genotyping for fecundity mutations.
- The replacements for nucleus males will be done from selected progeny born from nucleus parents and carrier of prolificacy mutated allele.

The remaining ewes can be improved genetically from 2 levels (nucleus to multipliers and multipliers to commercial tier).

Acknowledgements

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