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"Summary of a Doctoral (Ph.D.) Thesis"

APPLICATION OF MOLECULAR GENETIC METHODS FOR THE IDENTIFICATION OF BOOROOLA (FecB) MUTATION CARRIERS IN THE HUNGARIAN PROLIFIC MERINO BREED

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1. INTRODUCTION

The profitability of sheep breeding is determined by litter size. The establishment of the Hungarian Prolific Merino breed was one of the most important staps towards increasing lamb production of the Hungarian Merino population. The aim of the breeders was to establish a new breed with better lamb production than the Hungarian Merino, which would be capable of remaining in heat year around. In order to exploit the heterozis, this new breed could be suitable in cross breeding programmes as a mother line. The Hungarian Prolific Merino which is based on the Hungarian Merino x Booroola Merino crossing, was acknowledged as a new breed in 1992. The real value of the breed is that it carries the booroola (FecB) mutation. The method used for identifying the mutation carriers was complicated, time and labour consuming, which hindered its practical application.

With the knowledge of these facts, my aim was:

- to examine if the OarAE101 and BM1329 microsatellites are suitable for the selection of Fec^B carriers in the Prolific Merino breed at the level of population
- to examine the suitability of the OarAE101 and BM1329 microsatellites as markers for identification of Fec^B carriers in case of mutation introgression
- to compare the efficiency of the different genotyping methods (the traditional method based on the phenotype, and the molecular genetic method based on direct DNA test)
- to investigate the accuracy of the different methods
- to adapt the direct DNA test for genotyping using frozen sperm samples

2. MATERIALS AND METHODS

Ovulation rates examinations

Ovulation rate data were collected from Hungarian Prolific Merino ewes on the research farm of the University of Debrecen, Centre of Agricultural Sciences from 1998 - 2001. Determination of ovulation rate was performed as described by MAGYAR (1994). Ovulation rate was measured in natural oestrus cycle by laparoscopy in autumn.

Determination of marker genotype

Two microsatellites (OarAE101 and BM1329) were studied. Marker genotypes were determined in the Hungarian Prolific Merino population (as booroola mutation carriers; n=407) and in the Hungarian Merino sheep (as non carriers; n=46). At the beginning of my PhD study, these microsatellites were the closest linked known markers to the FecB locus. They were found to be suitable as markers for the introgression of the Fec^B allele into different sheep breeds. Examinations were carried out from 1998 - 2001.

DNA was isolated from blood samples of Hungarian Merino and from blood and frozen sperm samples of Hungarian Prolific Merino.

For genotyping, a polymerase chain reaction (PCR) was performed. Detection and visualisation of the microsatellite alleles were done with the ABI PRISM 310 Genetic Analyser, an automata laser detection system. “Genotyper” programme was used for data analysis.

Direct DNA test

DNA tests were carried out in autumn 2002 and in spring 2003. The same samples (from the Hungarian Merino and the Hungarian Prolific Merino) were used for the examination as were used for marker genotyping, and further samples were collected from the lambs born in 2002 in the Hungarian Prolific Merino population. Booroola genotypes of three Texel rams were also determined by the direct DNA test.

Detection of the mutation in the bone morphogenetic receptor type 1 B (BMPR-1B) gene (which is the FecB mutation itself) was performed by ACRS (Amplification Created Restriction Site) method. DNA was isolated from blood and sperm samples as well.

The notation of the different FecB genotype is the following:
BB – the individual carries the FecB mutation in homozygous from
B+ - the individual carries one copy of the mutant allele
++ - the individual does not carry the FecB mutation
The mutant allele of the FecB gene is also markable as Fec^B, and the wild type as Fec^+.

Statistical analyses

To investigate the effect of the marker genotype and the FecB genotype for the ovulation rate, oneway ANOVA was performed between marker genotype and phenotype and also between FecB genotype and phenotype. The Restricted Maximum Likelihood method was used for the calculation. The age, the year at the time of the OR measurement, and the marker genotypes were taken into the count as fix affects.

For the statistical examination of the difference in the marker allele frequency between the two populations and the distribution of the marker genotypes within the FecB genotypes, a Chi^2 probe was calculated. PIC (Polymorphic Information Content) was also calculated for the examination of the marker informativeness.

3. RESULTS

Results of examination of ovulation rate

The ovulation rate data of 64 pedigree ewes were involved into the variance analyses. Also the year and the age of the ewe were known at the time of the OR measurement. Furthermore, marker genotype and the results of the direct DNA test were also available for these animals. 115 ovulation rate data were collected altogether from the 64 ewes.

The distribution of the ovulation rate can be seen in Figure 1. The arithmetical mean of the OR is 3,12. 8,96% of the measured OR were 1, 33,91% were 2, and 57,39 were 4 or more indicating the presence of the FecB mutation in the population.

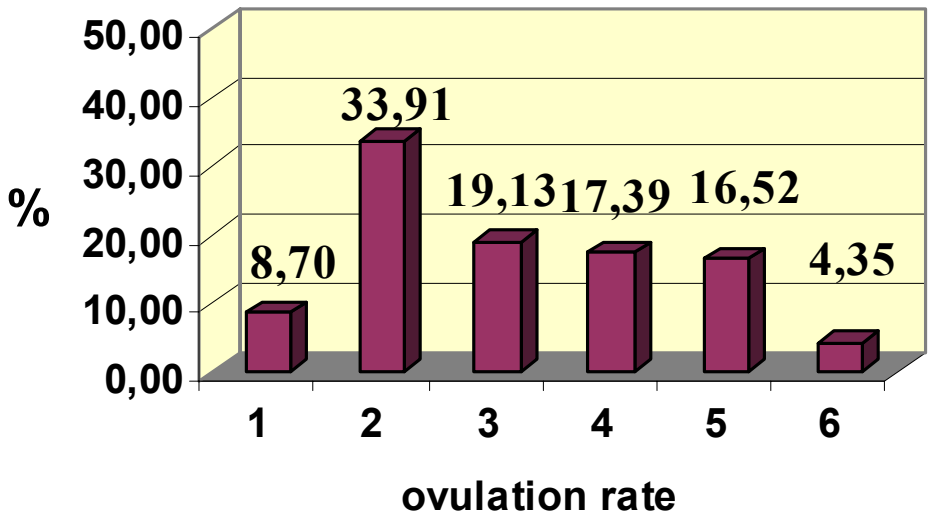


Figure 1. Distribution of ovulation rate of the Hungarian Prolific Merino ewes

Results of marker examinations

Alleles of microsatellites were named by their length, explained in base pairs.

Between the two breeds great differences were observed in the occurrence and frequency of the different alleles of microsatellites OarAE101 and BM1329 (Table 1. and Table 2.). The difference was found to be significant at the level of $P<0,001$.

Six alleles were identified in case of microsatellite OarAE101 but only three (87, 91, 99) were found in both populations. Three of them (105, 107, 109) were identified in the Hungarian Prolific Merino only. This suggests their Booroola origin.

Table 1. Allele frequencies of the OarAE101 microsatellite in the examined breeds

	Hungarian Prolific Merino			Hungarian Merino
	Whole population (1998-2003)*	Breeding animals (1998-1993)**	Ewes with OR data***	
N	**407**	227	64	**46**
Alleles				
87	**0,732**	0,785	0,82	**0,07**
91	**0,184**	0,133	0,08	**0,78**
99	**0,063**	0,062	0,06	**0,15**
105	**0,010**	0,009	0,01	**-**
107	**0,006**	0,009	0,02	**-**
109	**0,005**	0,002	0,01	**-**

*all individuals born and bred in the Hungarian Prolific Merino population in Debrecen in 1998-2003

**breeding animals in the Hungarian Prolific Merino population in Debrecen in 1998-2003

***64 Hungarian Prolific Merino ewes (which OR results were involved in the variance analyses)

Table 2. Allele frequencies of the BM1329 microsatellite in the examined breeds

	Hungarian Prolific Merino			Hungarian Merino
	Whole population (1998-2003)*	Breeding animals (1998-1993)**	Ewes with OR data***	
N	**407**	227	64	**46**
Alleles				
158	**0,706**	0,732	0,80	**0,152**
160	**0,149**	0,133	0,10	**0,435**
162	**0,066**	0,064	0,04	**0,196**
164	**0,076**	0,069	0,06	**-**
170	**0,003**	0,002	-	**0,174**
172	**-**	-	-	**0,043**

*all individuals born and bred in the Hungarian Prolific Merino population in Debrecen in 1998-2003

**breeding animals in the Hungarian Prolific Merino population in Debrecen in 1998-2003

***64 Hungarian Prolific Merino ewes (which OR results were involved in the variance analyses)

Also 6 alleles were obtained in the case of microsatellite BM1329. Four of them (158, 160, 162, 170) were presented in both breeds, but the allele 164 was identified in the Hungarian Prolific Merino only and the allele 172 was found only in the Hungarian Merino. The allele of 164 was identified in the case of one Booroola rams which suggest that Booroola origin.

Regarding the frequencies of the alleles, alleles 87 and 158 dominate in the Prolific Merino breed. The reason for this could be the linkage of these microsatellite alleles to the Fec^B allele in most families, since the breeders' aim was to increase the frequency of the FecB mutation in the breed. 51% of the rams were homozygous for both alleles (having 87/87 and 158/158 genotype).

Results of variance analyses between marker genotype and ovulation rate

The OarAE101 genotype was found not to have any significant effect for the ovulation rate.

Alleles 87 and 91 were presented in genotypes in which animals with high and low OR data were found. The group of 87/99 genotype had OR close to the population mean. In this group ewes were classified as ++ and BB genotypes respectively, which shows that the linkage phase between the Fec^B and marker alleles has changed in some families. Because of the recombination, there are some families where the marker alleles are linked to the Fec^B allele, and there are some families in which the marker alleles are linked to the wild Fec^+ allele (Figure 2.).

The BM1329 genotype showed significant effect on the number of corpoa lutea. Ovulation rate of animals with genotype 158/158 and 158/164 were found to be significantly higher than the animals with genotype 158/160 and 158/162 (Figure 3).

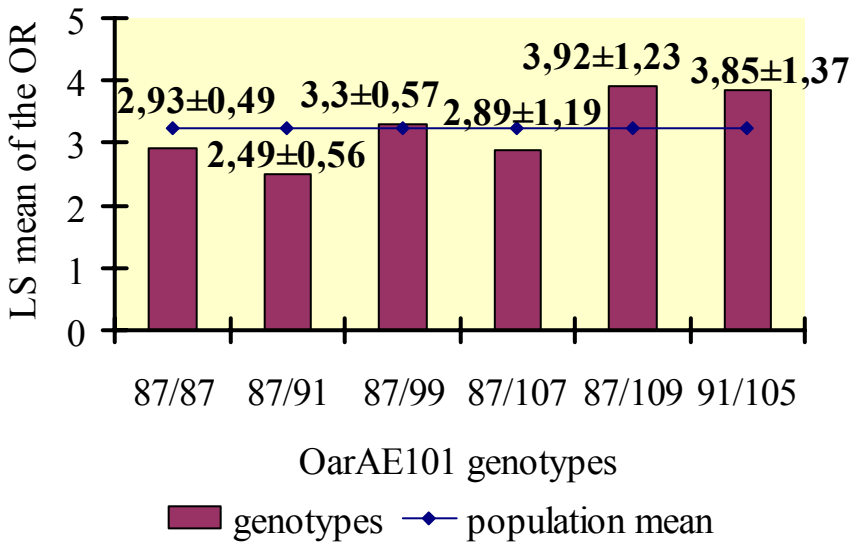
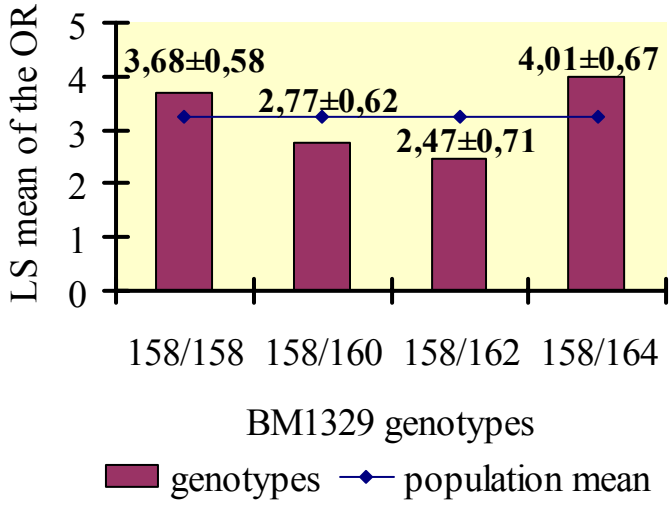


Figure 2. Distribution of ovulation rate of ewes plotted against the OarAE101 genotype



between 158/158 – 158/160 $\Delta S_{P<5\%} = 0{,}41$; 158/158 – 158/162 $\Delta S_{P<5\%} = 0{,}59$
between 158/160 – 158/164 $\Delta S_{P<5\%} = 0{,}62$; 158/162 – 158/164 $\Delta S_{P<5\%} = 0{,}79$

Figure 3. Distribution of ovulation rate of ewes plotted against the BM1329 genotype

Based on the results, alleles 158 and 164 are considered to be linked to the Fec^B allele and inherited together with the mutation in the Prolific Merino population. In some families linkage phase between the allele 158 of BM1329 microsatellite and the Fec^B allele has changed. The allele 158 was present in allele combinations (genotypes) such as 158/160, 158/162, here the animals have a „++” genotype, based on their OR data (Figure 3.).

The change of the linkage phase between the marker alleles and the Fec^B allele are justified by the recombination found in the Hungarian Prolific merino breed. 80 informative meiosis were found in the population and 13 of these were recombinant. The frequency of the recombination in the population is 0,1625.

The reason for the low number of meiosis is the high level of homozygozity for alleles 87 and 158. 34% of the population were found homozygous for both alleles.
PIC value of the markers were: $PIC_{BM1329=}$0,436, $PIC_{OarAE101}$=0,385.

Results of the direct DNA test

The results of the intensive research work which spanned five decades culminated in 2001 with the identification of the booroola mutation. Mutation on bone morphogenetic protein receptor – 1B was found to be associated with increased ovulation rate in Booroola Merino ewes by different research groups at almost the same time.
This discovery means there is no need for further phenotype (OR) measurement and marker examination. Determination of FecB genotype is possible right after birth, sex independently by direct DNA testing.

Results of variance analyses between FecB genotype determined by DNA test and the ovulation rate lived up to expectations. The FecB genotype was found to have a significant effect on OR at a level of P<0,001.

Table 3. The least square means of the different FecB genotypes

FecB genotype	Observation n	LS Means of ovulation rate	SD
++	11	1,70	±0,45
B+	51	2,59	±0,30
BB	53	3,97	±0,30

between BB and B+ $\Delta S_{P<0,1\%} = 0,60$; between ++ and BB $\Delta S_{P<0,1\%} = 1,024$

Differences were significant between the „BB" and „B+", and the „BB" and „++" genotypes respectively. Mutation has an additive effect on ovulation rate. In the case of the 64 Hungarian Prolific Merino ewes, the ovulation rate was increased by 0,89 by one copy of Fec^B allele. Homozygous carriers had an ovulation rate higher by 2,27 than the non-carriers.

Results of direct DNA test in case of ewes

Distribution of the present female Hungarian Prolific Merino population based on their FecB genotype determined by direct DNA test is the following: 37,41 % are BB homozygous, 43,88% are B+ heterozygous and 18,71% are ++ homozygous. The portion of animals carrying the Fec^B allele in a homo- or heterozygous form is 81,29%.

Results of direct DNA test in case of rams

FecB genotypes of 49 rams were determined. The distribution of the rams based on their FecB genotype can be seen in Table 4. Non-carriers were found in the case of the Booroola and the Hungarian Prolific Merino rams respectively. Unfortunately the numbers of the B+ heterozygous rams are relatively high in both breeds. The genotypes of the Texel rams were ++, as expected.

Table 4.: Distribution of the rams based on their FecB genotype

Breed	FecB genotype			Total
	BB	B+	++	
Booroola	5	8	2	15
Hungarian Prolific Merino	15	14	2	31
Texel	0	0	3	3

The accuracy of the direct DNA test is justified by the results of the repeated examination, which gave the same results.

Both blood and frozen sperm samples were available in case of six rams. DNA test performed using different samples gave the same result. This made it possible to accurately determine the FecB genotype of no longer living rams using only frozen sperm samples.

Comparison of the results of the direct DNA test and the marker genotypes

There is no need for a further marker test, since the FecB mutation was identified. However, what was supposed based on the marker examination could be justified by the results of comparison of marker genotypes and FecB genotypes determined by direct DNA test.

The distribution of the marker genotypes were found to be significant at the level of $P<0,001$ by the results of the Chi^2 probe.

As the distribution of marker genotypes shows the allele 87, 158 and 164 are linked to the Fec^B allele in most families in the Hungarian Prolific Merino population. However, this linkage was broken in some families because of the recombination which had occurred between the two marker loci, since animals having B+ and ++ genotypes were found in the groups of genotypes 87/87, 158/158 and 164/164, respectively. 88,2% of the animals with BB genotype were 87/87 homozygous, 77,6% of them 158/158 homozygous and 17,7% have 158/164 genotype (Table 5.).

The alleles 91 and 160 were found to be linked to the wild "+" booroola allele. They were the most frequent alleles in the Hungarian Merino breed. 17,3% of the animals with ++ genotype were 91/91 homozygous and 10% were 160/160. 80% of them carried the 160 allele in heterozygous form. Because of the recombination, there are some families in which these microsatellite alleles are linked to the Fec^B allele.

Table 5. Distribution of marker genotypes within the FecB genotypes (n=animals, numbers in the brecket indicate the frequency of marker genotypes based on FecB)

OarAE101 marker genotípus	FecB genotípus		
	BB n	B+ n	++ n
87/87	135 (0,65)	69 (0,33)	4 (0,02)
87/91	5 (0,04)	76 (0,7)	28 (0,26)
87/99	11 (0,27)	19 (0,48)	10 (0,25)
87/105	1 (0,17)	5 (0,83)	0 (0)
87/107	0 (0)	5 (1)	0 (0)
87/109	0 (0)	2 (1)	0 (0)
91/91	0 (0)	3 (0,21)	11 (0,79)
91/99	0 (0)	3 (0,60)	2 (0,40)
91/105	1 (1)	0 (0)	0 (0)
91/109	0 (0)	0 (0)	1 (1)
99/99	0 (0)	0 (0)	2 (0)
99/105	0 (0)	0 (0)	1 (1)
99/109	0 (0)	0 (0)	1 (1)

Calculated $Chi^2_{P<0,1\%}$= 215,83
From table $Chi^2_{P<0,1\%}$= 51,02
DF= 24

BM1329 marker genotípus	FecB genotípus		
	BB n	B+ n	++ n
158/158	118 (0,59)	71 (0,36)	10 (0,05)
158/160	2 (0,03)	52 (0,65)	26 (0,32)
158/162	2 (0,05)	29 (0,74)	8 (0,21)
158/164	27 (0,71)	11 (0,29)	0 (0)
158/170	0 (0)	1 (1)	0 (0)
160/160	0 (0)	1 (0,14)	6 (0,86)
160/162	0 (0)	1 (0,11)	8 (0,89)
160/164	1 (0,07)	12 (0,86)	1 (0,07)
160/170	0 (0)	0 (0)	1 (1)
162/162	0 (0)	1 (1)	0 (0)
162/164	0 (0)	3 (1)	0 (0)
164/164	3 (1)	0 (0)	0 (0)

Calculated $Chi^2_{P<0,1\%}$= 216,73
From table $Chi^2_{P<0,1\%}$= 48,3
DF=22

Comparison of the results of the direct DNA test and the FecB genotypes determined based on the ovulation rate

The FecB genotypes estimated based on ovulation rate and determined by direct DNA test were compared.

In the female population estimated FecB genotypes were available from 90 ewes. The FecB genotype was estimated to 80% accuracy and was overestimated in 20%. This explains the high frequency of the B+ heterozygous and ++ non-carriers in the Hungarian Prolific Merino population.

In the case of rams, FecB genotypes estimated by progeny test and determined by direct DNA test were available from 25 rams. Significant differences were found in genotypes determined by direct DNA test compared to the results from the progeny test or the parents' genotype.

The FecB genotypes of 7 rams imported from New Zealand were estimated based on the parents' genotype and the OR of the daughters. All the seven rams were classified as (BB) heterozygous carriers. Unfortunately, frozen sperm was available only from four rams, thus the genotype of these animals could be determined only by DNA test. One of the four rams was a homozygous carrier and 3 of them were genotyped as B+. These rams were used for a long time in the Hungarian Prolific Merino population.

Between 1998 and 2003, eighteen rams were classified for FecB genotype, based on the early OR of their daughters. Ovulation rates were measured in the 6th month of age of the daughters after PMSG treatment. The FecB genotypes of eleven rams of the eighteen were determined by the direct DNA test.

The same genotypes were determined by the breeders based on the daughter's phenotype in the case of five rams, as was shown by the direct DNA test. One ram was classified as homozygous although it was heterozygous, and five rams were classified as homozygous carriers, although they had B+ genotype. This means the FecB genotypes were overestimated in 45%, underestimated in 10%, and the genotypes were correctly estimated only in 45% of the cases.

A further 34 rams were genotyped for the FecB gene by direct DNA test. Three of them were Texel rams used as control (non carrier) animals. FecB genotypes of only eleven rams from the 31 were estimated by the breeders based on the OR of the daughters and the parents' FecB genotype. In the case of eight rams, the estimated FecB genotype corresponded with the results of the DNA test. The genotypes of the other two rams were overestimated by the breeders. Estimation of FecB genotype based on the OR of the daughters and the pedigree information was accurate in 80%.

Efficiency of the breeding programme for increasing the frequency of the Fec^B allele in the population was hindered by improper genotype classification.

The genotype of the 46 Hungarian Merino ewes and the three Texel rams were found to be ++. Examination of twenty B+ heterozygous and BB homozygous Prolific Merino ewes for FecB genotype was repeated and gave the same results in both cases, all which demonstrates the accuracy of the direct DNA test.

4. CONCLUSION

The following conclusion can be drawn from the results of molecular genetic examinations in the Hungarian Prolific Merino population.

Examination of ovulation rate

- Distribution of ovulation rate proves that the Hungarian Prolific Merino population carries the FecB mutation, since in more than 40%, the ovulation rate was 4 or higher.

Marker examination

- The difference in the frequency of microsatellite alleles between the Hungarian Merino and Hungarian Prolific Merino breeds was found to be significant at the level of $P<0,001$.

The microsatellite of BM1329 was found to have a significant effect on the ovulation rate at the level of P<0,5, but the microsatellite of OarAE101 was found to have no effect on the OR.

- Allele 87 of OarAE101 and allele 158 of BM1329 microsatellite are supposed to be inherited in linkage to the Fec^B allele in most families.
- Alleles 105, 107, 109 of OarAE101 and alleles 164 of BM1329 microsatellite have probably Booroola origin, since they were observed only in the Hungarian Prolific Merino breed.

In some families, the linkage phase has changed because of the recombination which occurred in the population. This conclusion is justified by the results of the comparison of FecB and marker genotypes. Distribution of marker genotypes within the FecB genotype were found to be significant at the level of P<0,001. This means that the distribution of the marker genotypes depends on the FecB genotype.

- It is not possible to determine the linkage phase of marker alleles and Fec^B allele at the level of population in the Hungarian Prolific Merino, only within families. The marker test is not suitable by itself for determination of the FecB genotype.

- Regarding to the mutation introgression programmes for increasing the litter size, results of marker test are found to be suitable for selection of Fec^B carriers in the recipient population.

Direct DNA test

- Mutation in the BMPR-1B receptor gene is proved to result in the high ovulation rate in the Hungarian Prolific Merino population, on the basis of the results of variance analyses between ovulation rate and FecB genotype, determined by direct DNA test.

- Distribution of the three FecB genotypes in the female and the male populations shows that the aim of the breeders has yet to be fulfilled. Presently, 40,4% of the whole population is homozygous BB.

- Great differences were found in the results of determination of FecB genotype by direct DNA test and estimation of the genotype based

on ovulation rate in the female population. FecB genotype was overestimated in 20% by the breeders. This could be the reason for the high frequency of non-carriers (++) and the heterozygous (B+) carriers in the female population.

- FecB genotype of rams based on the early ovulation rate of their daughters was estimated in a great differences comparing to the results of direct DNA test. It had 80% accuracy.

- FecB genotype of some of the rams was estimated based on the ovulation rate of their daughters measured in oestrus without synchronization. This method was found to be more accurate (80%) than the genotyping of rams based on the ovulation rate of daughters measured in oestrus after synchronization with PMSG. The latter one has 45% accuracy.

Because of overestimation of the level of the Fec^B allele carriers, the effort to increase frequency of the BB homozygous animals was not as successful as was expected.

- The direct DNA test was found to be completely reliable, based on the results of the repeated examinations.

- Accuracy of the direct DNA test performed using blood or frozen sperm samples was found to be the same.

5. NEW SCIENTIFIC RESULTS

1. Microsatellites (OarAE101 and BM1329) recommended in the international literature were found to be not suitable as markers for the selection of Fec^B carriers at population level in the Hungarian Prolific Merino breed, but could be suitable as markers in case of mutation introgression into the Hungarian Merino.

2. A direct DNA test worked out for identification of the mutation in the BMPR-1B gene was adapted and first used for selection of Fec^B carriers in Hungary. High prolificacy is proved to be caused by the mutation in the BMPR-1B gene by the results of direct DNA test.

3. In the female population, it was recorded that the FecB genotype can be determined with 79% accuracy and the mutation-carrying with 96,7%, based on the ovulation rate.

4. In the case of rams, accuracy of the determination of FecB genotype based on the ovulation rate of their daughters with and without PMSG treatment was 80% and 45%, respectively.

5. Accuracy of the direct DNA test was proved by repeated examination. The method was adopted for examination from frozen sperm samples, which gave the same results as blood samples. The direct DNA test is found to be suitable for determination of FecB genotype of the animals right after birth.

6. RECOMENDATION FOR THE PRACTICE

1. The OarAE101 and BM1329 microsatellites are not recommended as markers for the selection of Fec^B carriers in the Hungarian Prolific Merino breed, since the linkage phase between the marker alleles and the Fec^B allele are changeable among the families. They could be suitable as markers in the mutation introgression programmes, but their importance are pushed into the background by the discovery of the FecB mutation.

2. FecB genotypes of all the individuals bred and born in the Hungarian Prolific Merino population between 1998 - 2003 were determined by the direct DNA test. With the knowledge of the results, recommendations can be given for utilization of the breeding rams and for a mating plan.
   Selection of the lambs born in 2003 was performed based on the results of the direct DNA test. Favoured rams with ++ genotypes are recommended for exclusion from breeding. Accuracy of direct DNA test performed using blood and frozen sperm samples were found to be the same. Thus not longer living rams with BB genotypes from which we have frozen sperm samples are recommended for breeding in the future, through artificial insemination.

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