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APPLICATIONS AND PRACTICAL IMPLICATIONS OF ASSISTED REPRODUCTION AND MOLECULAR GENETICS TOOLS IN ACCELERATING GENETIC GAIN IN GOATS

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Abstract

Acceleration of genetic gain for some valuable traits represents for some local animal breeds an upmost necessity, especially when unimproved breeds are in competition with more productive ones. The progress in DNA and assisted reproduction technologies could represent a valuable tool to overcome these limitations. In many cases, valuable DNA mutations can have a low frequency, making the breeding process costly and time consuming. Therefore, artificial insemination can contribute to increase the frequency of these mutations in a population. We tested the viability of this combined approach on a Carpathian goat population. In this respect, the potential Carpathian goat males, candidates for semen collection needed for the artificial insemination experiments, were first selected from the herd book based on their known origin and phenotype. To confirm their parentage, we used a panel of 22 microsatellites markers. On the other hand we genotyped the remained candidate males for the alpha s1 casein gene, which is significantly associated with milk casein content and cheese yield. Only four males, with AA, BB and AB genotypes (positively associated with these traits) were retained for semen collection. A number of 450 Carpathian goat females were prepared for insemination using hormonal induction and synchronization of oestrus with fluorogestone acetate impregnated sponges (Chronogest sponges, with FGA 20 mg). In the 9th day after the sponges insertion, pregnant mare serum gonadotropin PMSG (Folligon) was injected in a dosage of 400 UI. The semen was collected from the selected males using an artificial vagina and was subsequentlyanalysed and processed for insemination. The goats were artificial inseminated in fixed point, 45 ± 2 hours after the sponges removal, with freshly diluted semen that was collected and diluted 2-3 h before insemination. Fecundity calculated after the end of parturition was 84.65%.

Key words*: artificial insemination, DNA markers, Carpathian goats.*

INTRODUCTION

In most European countries, there is an increased demand for high quality goat milk products. France is by far the most important producer of high-quality genetic stock and goat milk products. Saanen and Alpine goats are by far the most productive breeds, which suitable for intensive breeding. However, the main goat population, composed of unimproved local breeds, is located mainly in the Eastern and Southern European countries. Although are less productive, local goats could represent a viable solution to satisfy this increased demand for high quality goat milk products, due to the fact that they valorise a high-quality food from hilly pastures. Moreover, they can survive under harsh environmental conditions with low inputs, as compared with exotic breeds (Chemineau et al., 1997). It was shown that simple improvement of the technological parameters can lead to an increase milk yield and lactation period.

Genomic selection became in some farm species (Ex. cattle) an important tool to predict the breeding value of sires. In small ruminants the process is still very costly and in applied only at small scale and in some breeds with a high commercial value.

However, in some cases, the polymorphism of major genes can have a significant influence on phenotypes or production traits. Therefore, target genotyping of the animals for these specific markers, can have a beneficial influence in acceleration of the genetic gain for these traits. For example, selection of bucks

based on alfa S1 casein (*CNS1S1*) genotypes has already a long history in Alpine and Saanen goat breeds. In goat milk this protein normally represents around 32% from the whole casein fraction. The casein content of goat milk can significantly vary due to an increased polymorphism of *CNS1S1* gene, with at least 20 alleles currently known to date (Marletta et al., 2007; Bâlteanu et al, 2015). They are associated with four different expression levels *i.e.* strong alleles: A, B, C producing 3.5g α_{S1}-CN /l; intermediate alleles: E with 1.1g α_{S1}-CN /l; weak alleles: F with 0.45g αS1-CN /l and null alleles characterized by the absence of α_{S1}-CN in the milk of homozygous animals (Martin et al., 1999). Association studies highlighted a major positive effect of strong expression alleles on milk quality (casein content), rheological properties or cheese yield, as compared with week alleles and it is well documented in various breeds (Delacroix-Buchet, 1996; Caravaca et al., 2009; Yue et al., 2011; Bâlteanu et al., 2012).

When we talk about unimproved local breeds, Romania can be a suggestive example. Currently the goat population accounts for 2 million heads. The majority of these goats (about 90%) are represented by Carpathian breed. Indeed, improving milk production traits by crossing with improved breeds can represent a convenient solution for commercial farms. But, in many cases this is not a solution since the preservation of local genetic stock is essential.

Acceleration of the genetic gain in Carpathian goat, especially for milk production traits, represents an upmost necessity. In Carpathian goat it was shown that the variability in milk casein contents and cheese yield can be significantly affected by a high frequency of defective E and F alleles (Bâlteanu et al., 2012). They have an estimated frequency of around 45% in this breed (Bâlteanu et al., 2015).

Artificial insemination can significantly contribute to increase the frequency of strong expression *CSN1S1* alleles (A or B), with a beneficial effect of milk casein content and cheese yield. We tested the viability of this combined approach in some Carpathian goat populations.

MATERIALS AND METHODS

Bucks selection, blood sampling, parentage testing and CSN1S1 genotyping

The potential Carpathian bucks candidates for semen collection needed for the artificial insemination experiments, were first selected from the Carpathian breed herd book, based on their known origin and phenotype. A number of 36 males between 8 to 40 months of age were first selected from six farms. Blood samples were collected in K_3-EDTA coated tubes from jugular vein. Additionally, to verify the parentage, blood samples were collected from their presumed parents.

DNA samples were purified from 100µl whole blood using Quick-gDNA MiniPrep kit and according to the manufacturer instructions (Zymo Research Corporation, USA). The DNA concentration and purity was determined on a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA).

To confirm their parentage we used a panel of 22 microsatellites markers (including the recommended ISAG panel). Amplified multiplex PCR reactions were analysed in Applied Biosystems 3500 device. Parentage verification was done by comparing the size of the generated microsatellites fragments for each marker, using GeneMapper software (Applied Biosystems, USA).

Genotyping of candidate males for the A, B, E or F *CSN1S1* alleles was done by PCR-RFLP and AS-PCR. Two distinct PCR reactions were prepared for each goat.

To discriminate between A, B/E and F, a small polymorphic fragment containing the entire exon 9 and partial regions from introns 8 and 9 was amplified in 25 µl final volume reaction containing 1X Tissue Green PCR Master Mix (Fermentas, Lithuania), 10 pmol of each primer and 50 ng of genomic DNA. The thermal profile was as follows: 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 58 °C for 1 min, 72°C for 1 min and a final extension step of 72°C for 7 min. Amplicons were digested with 10 U of *XmnI* endonuclease (Thermo Scientific, USA) for 15 min at 37°C and analysed on a 3% agarose gel stained in containing 1X GelRed.

To discriminate between E allele from non-E alleles, DNA samples were amplified in the same conditions, but using a specific primer set flanking a region from exon 19th, were a 457 bp LINE element specific to E allele can be inserted or not.

Oestrus synchronisation, semen collection and artificial insemination

A number of 450 Carpathian goat females from four distinct farms located Tulcea County were prepared for insemination.

Hormonal induction and synchronization of oestrus was done with fluorogestone acetate impregnated sponges (Chronogest sponges, with FGA 20 mg). In the 11th day after sponges insertion they were removed and pregnant mare serum gonadotropin (Folligon) was injected in a dosage of 400 UI.

Several hours before artificial insemination, the semen of four males, selected based on genetic criteria (parentage and *CSN1S1* genotypes), was collected using artificial vagina. Subsequently the semen was evaluated and prepared for artificial insemination of selected females.

The goats were inseminated in fixed point, 45 ± 2 hours after sponges removal, with freshly diluted semen that was collected and diluted 2-3 h before insemination. The fecundity was calculated after the end of parturition.

RESULTS AND DISCUSSIONS

Parentage verification of the candidates bucks was performed based on the genotypes obtained for the 22 microsatellites, by comparing them with the genotypes of the presumed parents.

Allelic sizes for some of these markers are highlighted as an example for two presumed families in Table 1.

Table 1. Allelic sizes obtained for five microsatellites markers is two presumed Carpathian goat families; F1= family 1, M=mother; F=father; O=offspring

Specie	Breed	Tag	Sex	ID	Microsatellite markers (size in base pairs)										
					BM1329 allele 1	BM1329 allele 2	BM1818 allele 1	BM1818 allele 2	CSRD247 allele 1	CSRD247 allele 2	HSC allele 1	HSC allele 2	MM12 allele 1	MM12 allele 2	n.....
Capra hir	Carpathia	RO2565	F	F1-M	173	181	254	258	234	242	278	294	98	114	.....
Capra hir	Carpathia	RO2565	M	F1-F	171	171	258	268	230	232	280	286	104	104	.....
Capra hir	Carpathia	RO2551	M	F1-O	169	181	254	258	230	234	278	292	98	98	.....
Capra hir	Carpathia	RO2560	F	F2-M	171	177	254	268	230	234	278	300	94	106	.....
Capra hir	Carpathia	RO2560	M	F2-F	169	177	260	260	234	238	272	293	98	98	.....
Capra hir	Carpathia	RO2560	M	F2-O	169	171	254	260	230	234	293	300	98	106	.....

In the first presumed family (F1), the comparative analysis of fragment sizes, obtained in all microsatellites markers of the offspring (F1-O) and its presumed mother (F1-M), shown 100% compatibility. In contrast, the presumed father (F1-F) of the offspring (F1-O) showed incompatibility for several markers. For example, the presumed father is homozygous for the BM1329 marker (171 bp), fragment which is not present in the offspring. The same situation was found for other markers (ex: HSC, MM12 etc; Table 2).

In the second family (F2) we observed 100% compatibility between the offspring and its parents.

Only the goats that passed the parentage test were further keept as potential candidates for semen collection and were further submitted to *CSNS1* genotyping.

The identification of the main *CNS1S1* genotypes of the analysed bucks was done base on the correlation of the electrophoresis profiles obtained for the two distinct DNA tests performed for each goat.

The first PCR-RFLP test allows the discrimination between A, B/E and F allele. In the case of A allele there is a 11 bp deletion located in intron 9, which is absent in B/E and F allele.

Digestion of the fragment amplified from A allele with *Xmn*Iendonuclease generates two fragments of 150bp and 63bp. In the case of B/E alleles, which are similar in this amplified region, one of the fragments 11bp longer (161 bp), as compared with that obtained in the case of A allele.

Furthermore, the deletion of the 23rd nucleotide (a cytosine) from exon 9 in the case of F allele,

abolishes the *XmnI* restriction site, generating an undigested fragment.
The second AS-PCR testallowed discrimination between E and non-E alleles, based on the 457 bp LINE element insertion from the 19th exon that is characteristic to E allele.

Different combinations of these patterns that correspond either with homozygous or heterozygous genotypes and are highlighted in Figure 1.
Allele and genotypes frequencies at the CSN1S1 locus were calculated for in this bucks population (Table 2).

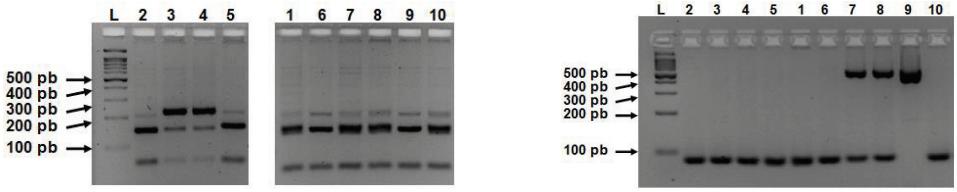

Figure 1. Identification of CSN1S1 genotypes by PCR-RFLP (left pictures) and AS-PCR (right pictures). Samples genotypes 1-AB; 2-AA; 3-AF; 4-AF; 5-BB; 6-BB; 7-AE; 8-AE; 9-EE; 10-AB

Table 2. Allelic frequency at *CSN1S1* in the analyzed Carpathian goat bucks population

Locus	No. of candidate bucks	Allele frequency				Genotypes frequency								
CSN1S1	36	A	B	E	F	AA	AB	BB	EE	FF	AE	BE	AF	BF
		0.35	0.40	0.07	0.18	0.17	0.25	0.14	0.03	0.03	0.03	0.06	0.08	0.22

The cu frequency of defective allele (E and F) was much lower (0.25) compared with strong expression allele (0.75). However, we found a significant frequency of genotypes carriers of at least one defective allele (0.39).
The cumulated frequency of strong genotypes (AA, AB and BB), significantly associated with higher milk casein content and cheese yield, was 0.56. Only four males from this category were further retained for semen collection.
The synchronization of oestrus and ovulation was accomplished using a hormonal treatment protocol based on progesterone and prostaglandins.
Hormonal treatment is necessary if artificial insemination is used either in natural or out of season oestrus induction. According to literature the use of one dose of 20mg of FGA can lead to a higher rate of kidding than the use of other doses (Barbosa et al., 2009).
The results obtained in the current study based on the cervical opening degree showed that the females acquired a synchronized oestrus and the average percent of non-returned to oestrus goats after two oestrous cycles was 91.28%.
The rate of females with third degree cervical opening (intrauterine deposition of sperm) was 51%, second degree 38% and first degree 10%.
The reproduction indices in she-goats used for artificial insemination during induced oestrus are shown in Table 3.
In the first farm we registered a 88 % rate of non-returning goats to oestrus after two cycles, a fecundity of 82% and a prolificacy 173.4% in goats inseminated with freshly collected and diluted semen.
In the second farm the rate of non-returning goats was 92%, with a fecundity of 88% and prolificacy 168.2%.
In the third farm the rate of non-returning goats was 88%, with fecundity rate of 79% and prolificacy 170.23% and in the fourth farm these indices were 92,5%, 84,81% and 170.98%, respectively.

Table 3. Reproduction indices of Carpathian she-goats used for artificial insemination

Group	No. of inseminated goats	% of goats non-returned to oestrus	Fecundity %	Prolificacy %
Farm I	90	88.63	82.25	173.40
Farm II	90	90.00	85.55	168.88
Farm III	150	94.00	86.66	170.66
Farm IV	120	92.50	84.16	191.66

Similar results using an artificial insemination protocol with diluted semen were obtained in other studies (Faigl et al., 2012).

According other studies, there is a direct connection between the fecundity and semen deposition, uterine insemination being associated with a higher fecundity rate.

The results obtained in our study point out that the uterine deposition (third degree cervical opening) of the semen is associated with a higher kidding rate, as demonstrated in other studies (Salvador et al., 2005).

CONCLUSIONS

In this study we tested the possibility to use DNA and assisted reproduction technologies to spread valuable mutations associated with milk traits in goats. We tested the viability of this combined approach in four populations belonging to unimproved Carpathian goat breed.

The potential candidate bucks for semen collection were submitted for parentage testing and for *CNS1S1* genotyping.

Only four males with confirmed genetic origin and valuable *CSN1S1* genotypes (associated with higher milk casein content and cheese yield) were used for the artificial insemination of 450 oestrus synchronised she-goats.

The high fecundity rate of oestrus synchronised goats calculated after the end of parturition was registered (84.65%).

By using freshly collected semen exclusively from *CSN1S1* genotyped goats we proved the viability of this model to spread valuable genes at a higher rate in Carpathian breed.

In this particular case this combined approach can substantially contribute to the improving of casein contents and cheese yield and at the same time to the preservation of the valuable gene pool of this local breed.

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