Phenotypic and genetic characterization of the Romanian Grey Steppe breed

Objectives:

1. ***Elaboration of the specialized documentation and the specific lab techniques;***
2. ***Studies regarding the quantitative and qualitative features of milk from the groups under study;***
3. ***Characterization of lactoprotein genes (β-casein, k-casein, β-lactoglobulin) for the analysed individuals;***
4. ***Making up the animal groups depending on the determined genotypes;***
5. ***Determination of the blood groups and establishing the individuality of the analysed individuals;***
6. ***Making the karyotype of the analysed individuals.***

The research project focuses on the monitoring of the Romanian Grey Steppe nucleus from Moldavia in order to characterize it from the phenotypic and genetic viewpoint, data that will be used for the breed preservation.

The activities carried out within the research project for 2008 focused on:

- ✓ The specialized documentation, elaboration of the conceptual model;
- ✓ Establishing the models for the work methods;
- ✓ Quantitative determinations of milk for the studied individuals, *statistic determinations;*
- ✓ Qualitative determinations of milk for the studied in;
- ✓ Harvesting and sending off the blood and milk samples, *documentation stage of the research tem in a prestigious lab for the determination of the molecular markers;*
- ✓ Determination of genotypes of the bovine lactoproteins for the Romanian Grey Steppe breed and establishing the relations of such genotypes with the morphoproductive and resistance features of the breed;
- ✓ Study of the obtained genotypes and establishing their dynamics based on the laws specific to panmictic populations;
- ✓ Publishing the partial results obtained;
- ✓ Elaboration of the specialized documentation in order to analyse the blood groups;
- ✓ Analysis of the blood groups of the individuals under study;
- ✓ Elaboration of the specialized documentation in order to make the karyotype;
- ✓ Analysis of karyotype for the individuals under study.

The Romanian Grey Steppe breed, on the verge of extinction, has been included in a preservation programme of animal genetic resources and it is being raised in a reduced nucleus at the Research Development Station for Bovine Growing – Dancu Iaşi *(figure 1).*

We do not have more recent data about the Romanian Grey Steppe in terms of productive characters and current genetic value *and this is the reason why our team has started researching the nucleus existing at Dancu Station.*

Fig. 1 Romanian Grey Steppe breed from S.C.D.C.B. Dancu Iaşi

STUDIES REGARDING THE QUANTITATIVE AND QUALITATIVE FEATURES OF MILK FROM THE ANALYSED GROUPS BELONGING TO THE ROMANIAN GREY STEPPE BREED FROM S.C.D.B. DANCU IAŞI

When speaking of milk quality, one must take into account both its physical-chemical features and the organoleptic ones and also a series of indicators such as: bacterial and biological level (number of bacteria and number of somatic cells); inhibiting substances such as impurities, disinfectants, antibiotics etc.

The quality criteria for the raw milk are established in Ch. I from Section IX,, Annex III of the (EC) Regulation no. 853/2004. Since the restructuring of the agro-alimentary units has

been one of the problematic issues for Romania's accession to the E.U., the European Committee has granted the milk processing units a transition period up to 31.12.2009 so that they might harmonize with the community hygiene requirements.

From the qualitative viewpoint, the cow mil must have *the standard fat content of 3.5 – 3.7%, protein 3.2 %, minimal density of 1.027, acidity 14-16°T,* temperature should not exceed 4°C and the impurification level should comply with the accepted limits. Free acidity of milk is expressed by pH which shows the hydrogen ion concentration from solutions. The cow milk has a pH between 6.7-6.4. *The freezing point -0.555^{o}C is the most constant feature of milk.* The indicators related to the bacterial and biological level of milk are given in table 1.

Table 1

Milk quality in the European Union in terms of somatic cell content, the total number of germs and butyric spores

Specification	***Current situation***	***Objectives***
NCS/ml	‹ 400,000	‹ 250,000
NTE/ml	‹ 100,000	‹ 50,000
Butyric spores/ml	‹ 1,000	‹ 1,000

In table 2 we present the average values and the variability indices for milk yield by successive lactations for the Romanian Grey Steppe breed. As we may see, the length of total lactation is also the length of normal lactation, since the period of lactation of 305 days is not exceeded. The milk quantity per lactation varied between *1589.64 kg (1st lactation) and 2535.43 kg in the 5th lactation* which is also the maximum lactation. Starting from the 6th lactation, the milk quantity decreases and in the 8th lactation it reached the value of 1078.5 kg.

In the first lactation 62.69 % of the maximum lactation was achieved, a value highlighting the tardiness of the Romanian Grey Steppe breed in terms of milk yield.

The variability of the milk quantitative production is very strong, the values of standard deviation ranging between *s = 544.10 kg of milk in the 1st lactation and 1185.89 kg of milk in the 5th lactation*, and the *variability coefficients between V % = 36.43 and V % =46.77*. The very strong variability of the nucleus under study proves the lack of selection according to this basic parameter and the possibility of genetic melioration by withholding and multiplying the valuable genotypes. Mention must be made of the fact that in the nucleus under study there were individuals with a maximum production of 4080 kg or 3080 kg of milk per lactation.

In the genetic structure of the livestock under study, we identified three groups of paternal semi-sisters with productions of 1548.22 kg (code 79009) and 1752.33 kg (code 79005), quite low values in terms of milk quantity.

Table 2

Average values and variability of milk production indices, by successive lactations, for the Romanian Grey Steppe breed

Specification	Sample statistics	Total lactation					Normal lactation				
		Milk kg	% Fat	Kg fat	% prot	Kg prot	milk Kg	% fat	Kg fat	% prot	Kg prot
1st lactation	n	30	30	30	30	30	30	30	30	30	30
	$\overline{X}$	1589.64	4.64	68.94	3.53	49.95	1589.64	4.64	68.94	3.53	49.95
	$\pm s_{\bar{x}}$	102.82	0.09	4.67	0.053	3.447	102.82	0.09	4.67	0.053	3.447
	s	544.10	0.49	24.74	0.242	15.794	544.10	0.49	24.74	0.242	15.794
	V%	36.43	11.21	37.48	6.86	31.60	36.43	11.21	37.48	6.86	31.60
	Min	360	3.40	15.00	3.07	27	360	3.40	15.00	3.07	27
	Max	2612	5.30	107.00	3.91	77	2612	5.30	107.00	3.91	77
2nd lactation	n	27	27	27	27	27	27	27	27	27	27
	$\overline{X}$	1699.96	4.65	67.04	3.56	54.65	1699.96	4.65	67.04	3.56	54.65
	$\pm s_{\bar{x}}$	147.15	0.09	5.09	0.053	3,.915	147.15	0.09	5.09	0.053	3,.915
	s	705.71	0.45	24.42	0.236	17.509	705.71	0.45	24.42	0.236	17.509
	V%	41.58	9.88	33.43	6.62	32	41.58	9.88	33.43	6.62	32
	Min	198	3.70	10.00	3.08	17	198	3.70	10.00	3.08	17
	Max	3565	5.40	111.00	3.90	99	3565	5.40	111.00	3.90	99
3rd lactation	n	20	20	20	20	20	20	20	20	20	20
	$\overline{X}$	2092.80	4.51	93.00	3.59	64.75	2092.80	4.51	93.00	3.59	64.75
	$\pm s_{\bar{x}}$	215.08	0.12	8.76	0.053	3.915	215.08	0.12	8.76	0.053	3.915
	s	833.01	0.49	33.95	0.236	17.509	833.01	0.49	33.95	0.236	17.509
	V%	39.80	10.85	36.50	6.82	31.73	39.80	10.85	36.50	6.82	31.73
	Min	434	3.50	22.00	3.18	29	434	3.50	22.00	3.18	29
	Max	4080	5.30	144.00	3.90	99	4080	5.30	144.00	3.90	99
4th lactation	n	15	15	15	15	15	15	15	15	15	15
	$\overline{X}$	2082.10	4.62	91.10	3.65	70.51	2082.10	4.62	91.10	3.65	70.51
	$\pm s_{\bar{x}}$	250.46	0.13	9.71	0.064	9.237	250.46	0.13	9.71	0.064	9.237
	s	792.03	0.41	30.72	0.222	31.998	792.03	0.41	30.72	0.222	31.998
	V%	38.04	8.94	33.72	6.09	45.40	38.04	8.94	33.72	6.09	45.40
	Min	835	4.10	45.00	3.21	30	835	4.10	45.00	3.21	30
	Max	3080	5.30	138.00	4.09	130	3080	5.30	138.00	4.09	130

Table 2 (continuation)

Specification	Sample statistics	Total lactation					Normal lactation				
		Milk kg	% fat.	Kg fat	% prot	Kg prot	Milk kg	% fat.	Kg fat	% prot	Kg prot
5th lactation	**n**	8	8	8	8	8	8	8	8	8	8
	$\overline{X}$	2535.43	4.73	119.92	3.71	69.14	2535.43	4.73	119.92	3.71	69.14
	±s $\bar{x}$	448.22	0.21	25.25	0.038	6.753	448.22	0.21	25.25	0.038	6.753
	s	1185.89	0.57	66.82	0.10	16.541	1185.89	0.57	66.82	0.10	16.541
	V%	46.77	12.06	54.01	2.69	37.60	46.77	12.06	54.01	2.69	37.60
	Min	675	3.70	32.00	3.59	25	675	3.70	32.00	3.59	25
	Max	4087	5.30	212.00	3.88	95	4087	5.30	212.00	3.88	95
6th lactation	**n**	5	5	5	5	5	5	5	5	5	5
	$\overline{X}$	1411.00	4.95	69.00	3.58	39	1411.00	4.95	69.00	3.58	39
	±s $\bar{x}$	201.67	0.15	9.28	0.103	7.348	201.67	0.15	9.28	0.103	7.348
	s	403.35	0.31	18.56	0.253	18	403.35	0.31	18.56	0.253	18
	V%	28.58	6.28	26.90	7.06	46.20	28.58	6.28	26.90	7.06	46.20
	Min	818	4.60	43.00	3.08	22	818	4.60	43.00	3.08	22
	Max	1705	5.30	87.00	3.79	65	1705	5.30	87.00	3.79	65
7th lactation	**n**	3	3	3	3	3	3	3	3	3	3
	$\overline{X}$	1519.00	4.66	83	3.54	68	1519.00	4.66	83	3.54	68
	±s $\bar{x}$	270.46	0.13	9.71	0.038	6.753	270.46	0.13	9.71	0.038	6.753
	s	792.03	0.41	30.72	0.10	14.541	792.03	0.41	30.72	0.10	14.541
	V%	18.04	3.94	13.72	2.29	27.20	18.04	3.94	13.72	2.29	27.20
	Min	835	4.10	45.00	3.49	66	835	4.10	45.00	3.49	66
	Max	1980	5.30	138.00	3.58	71	1980	5.30	138.00	3.58	71
8th lactation	**n**	2	2	2	2	2	2	2	2	2	2
	$\overline{X}$	1078.5	5.28	57.44	3.71	39	1078.5	5.28	57.44	3.71	39
	±s $\bar{x}$	268.46	0.13	9.71	0.027	5.325	268.46	0.13	9.71	0.027	5.325
	s	792.03	0.41	30.72	0.15	12.72	792.03	0.41	30.72	0.15	12.72
	V%	12.04	2.94	10.72	2.14	15.10	12.04	2.94	10.72	2.14	15.10
	Min	875	5.19	45.00	3.69	39	875	5.19	45.00	3.69	39
	Max	1282	5.30	68.00	3.73	39	1282	5.30	68.00	3.73	39

As for the qualitative features of milk, the fat percentage reaches the maximum value in the 5th lactation, namely 4.71%. The same evolution may also be noticed for the protein percentage that in the 5th lactation reaches 3.71%. The variability of the indicators mentioned above is intermediate to high (V = 6.09 – 12.06 %) offering a good possibility for the improvement of population by selection in terms of qualitative indicators of milk yield.

The fat and protein quantity has a similar evolution to that of milk production due to the tight relation existing between these characters (r_{pg} = 0.75 – 0.99).

We must also mention the high variability of the specified indicators highlighting the heterogeneity of the nucleus under study.

The variability of the qualitative indicators for milk yield is analysed in the research project and it is succinctly presented below *(table 3 and fig. 2).*

As for the percentage of milk protein, the module of population has a relative frequency of 26.92 % and belongs to the class 3.54 – 3.66 % protein. The plus variants enter the module class and the classes higher than the module (3.67 - 3.78% and 3.79 - 3.91% protein).

Table 3

Variation series for milk protein percentage in the 1st lactation

Class	***Class interval***	***Absolute frequency***	***Relative frequency***
1	3.03 – 3.16	4	15.38
2	3.17 – 3.28	2	7.69
3	3.29 – 3.41	3	11.54
4	3.42 – 3.53	2	7.69
5	3.54 – 3.66	7	26.92
6	3.67 – 3.78	6	23.08
7	3.79 – 3.91	2	7.69

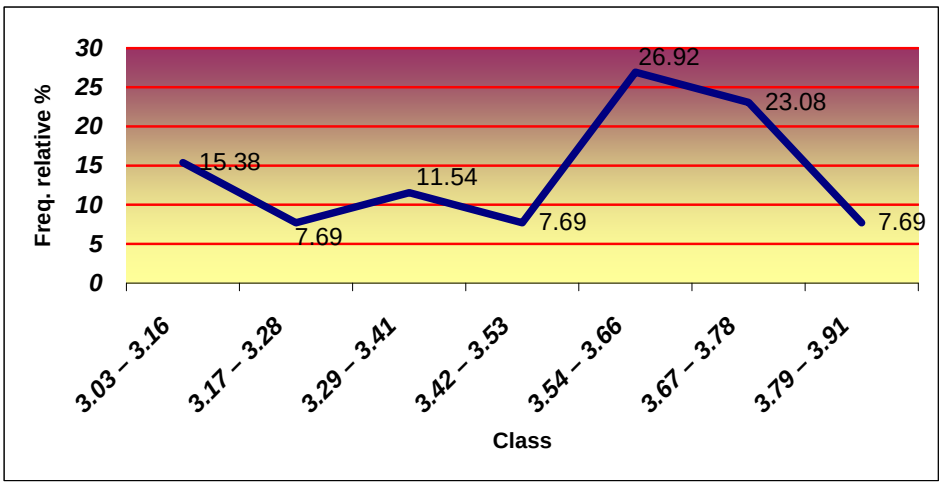

Fig. 2 Polygon of frequencies for milk protein percentage in the 1st lactation

CHARACTERIZATION OF LACTOPROTEIN GENES (B-CASEIN, K-CASEIN, B-LACTOGLOBULIN), FOR THE INDIVIDUALS UNDER STUDY

The study of polymorphism of milk proteins for the members of the Bovidae family from the Romanian Grey Steppe breed was effectuated by PCR-RFLP technique, and for the study of polymorphism of all bovine lactoproteins we also used the isoelectric focusing technique (IEF).

Use of PCR-RFLP technique

To extract the DNA from the white cells of integral blood, we used the WizardGenomic DNA Purification Kit (Promega)

To identify the genotypes of the two loci under study (*k-casein and β-lactoglobulin)*, we used the protocols described by Medrano and Aguilar–Cordova (1990). They allow the identification of alleles A and B that are most frequently met in the members of the Bovidae family populations.

PCR amplification:

The identification of alleles A and B from ***K-casein*** locus relies on the amplification of a product of 350 pb from the gene by means of two sense and antisense primers: JK 5: 5'- ATC ATT TAT GGC CAT TCC ACC AAA G –3'; JK3: 5'- GCC CAT TTC GCC TTC TCT GTA ACA GA – 3'.

The dilution of primers was made by sterile distilled water with a concentration up to 10 picomoles/μl. For a reaction volume of 25μl, we used 24 μl of PCR master mix (with an optimal composition resulted from the diverse combinations attempted) and 1μl of undiluted DNA (with concentration between 105.9-556.9 ng/μl depending on the sample).

The samples were amplified in a MyGene thermocycler using the following conditions:

1. DNA predenaturation: 94°C/3 minutes - **1 cycle**

2. Denaturation: 94°C/1 minute
3. Primer fixation: 60°C/1 minute
4. Extension (Elongation): 72 °C/ 1 minute
} **35 cycles**

5. Final extension: 72°C/7 minutes- **1 cycle**

The amplified samples were **stored** for 24 hours at 4°C.

To obtain an amplification product of 262 pb from the gene of ***β- lactoglobulin***, so as to identify alleles A and B, we used two sense and antisense primers: BLGP3: 5'-GTC CTT GTG CTG GAC ACC GAC TAC A–3'; BLGP4 :5'- CAG GAC ACC GGC TCC CGG TAT ATG A–3'.

The dilution of primers was made by sterile distilled water with a concentration up to 10 picomoles/μl. The reaction volume and the amplification cycles were identical to those used for K-casein.

Digestion of amplification products:

The identification of alleles A and B of K-casein based on the enzymatic digestion of this fragment of 350 pb (part of exon IV and intron IV) was possible based on some mutations differentiating the two alleles in this area. Thus, **in case of allele A**, ACC codon codifies treonine from position 136 of mature protein, and GAT codon codifies the aspartate amino acid from position 148. In case of variant B, a cytosine from ACC codon is replaced by a thymine transferring in ATC codifying the isoleucine from position 136 of the mature position. Also **in variant B** the replacement of adenine by cytosine in GAT codon makes the newly-formed codon CGT codify another amino acid in position 148, namely alanine. Consequently, mutation GAT-GCT, makes the restriction site of Hinf I enzyme from this position disappear in variant B. Based on this substitution, the two alleles may be differentiated by Hinf I digestion.

For a volume of 25 μl produced by amplification from K-casein gene, we added 10μl of restriction mixture containing: 0.8μl Hinf I (8 units), 6.2μl sterile deionized water, 3μl NEB 2 buffer.

The identification of alleles A and B of β-lactoglobulin was made by the same principles. The protean variants A and B differ by two substitutions of amino acids caused by two substitutions of nucleotides from the gene structure. **In case of allele** A, in the polypeptidic chain in position 64 there is aspartate (codified by GAT codon) and valine in position 118 (codified by GTC codon). **Incase of allele B**, in the polypeptidic chain, in position 64, aspartate is replaced by glycine (codified by GGT codon), and in position 118 instead of valine we encounter alanine (codified by GCC codon). The substitution of thymine from GTC codon from allele A by cytosine (resulting GCC codon) in allele B created a new restriction site for the restriction enzyme Hae III. This substitution is placed on the fragment of 262 pb amplified (part of exon IV and intron IV), making possible the differentiation of the two alleles.

For a volume of 25 µl produced by amplification from β-lactoglobulin gene, we added 12.5µl of restriction mixture containing: 0.6µl Hae III (6 units), 8.15µl sterile deionized water, 3.75µl NEB 2 buffer.

The samples were incubated at 37 °C for 3 hours in case of K-casein and over the night for β-lactoglobulin so that the amplification products should be completely digested. Digestion was stopped by adding 7µl loading buffer containing 500 µl TBE 10X (TBE, pH=8,3), 400 µl glycerol and 100 µl Bromfenol Blue 2%.

Identificaiton of alleles A and B of K-casein and β-lactoglobulin by migration in agarose gel:

Taking into account the size of the fragments expected after digestion (between 84-266 pb in case of K-casein, and 74-153 pb in case of β-lactoglobulin), we prepared an agarose gel with 3% concentration. This allowed a good separation of the fragments obtained following the digestion. To prepare 100 ml of gel with 3% concentration, we weighed 3 g of agarose to which we added TBE 1X up to 100 ml. After a short homogenization, the solution was transferred in an Erlenmayer glass and then heated on an electric stove with magnetic stirrer until it became homogenous and clear. After a preliminary cooling (not full), we added 8 µl of ethidium bromide (work solution), and after a short homogenization, the gel was poured into the electrophoresis tank and left for polymerization for 30 minutes.

The samples were loaded in cups, in the first cup introducing a DNA ladder of 123 pb and in the second the undigested amplification product of 350 pb from K-casein gene. In case of β-lactoglobulin, in the second cup we introduced the undigested amplification product 262pb.

Electrophoresis took place in TBE 1X buffer (pH= 8.3), at a voltage of 70V for 2 hours. The examination of the gel and taking of the picture were made in UV light.

Results obtained:

DNA purity and concentration were measured by spectrophotometry. Purity ranged between 1.20-1.54 and the DNA quantity extracted from 200µl of integral blood was between 105.9-556.9 ng/µl. The DNA samples were not purified, from every sample we used 1µl of raw DNA (undiluted) for amplification.

The use of DNA outside its purity considered optimal (between 1.7-1.8) and of a larger quantity than the one specified in the specialized literature (between 30-100ng/µl), did not affect the

amplification or digestion processes. This is proved by the presence in gel of a large quantity of amplification product and its full digestion achieved by Hinf I and Hae III enzymes.

The identification of alleles A and B of K-casein was possible by the amplification of a fragment of 350 pb, located between exon IV and intron IV, using specific primers (JK3, JK5). This fragment includes 201 pb from exon IV and 149 pb from intron IV *(fig. 3)*.

The DNA fragment of 350 pb amplified from allele A contains two restriction sites for Hinf I enzyme, one in position 134 and another in position 266, resulting three types of fragments of 132 pb, 134 pb, 84 pb, after the digestion with this enzyme. They correspond to genotype AA of this locus. Due to an almost similar size and the impossibility for their separation in the agarose gel, the two fragments of 132 pb and 134 pb migrated together and they appeared under the form of one single strip containing a mixture of these two fragments *(fig. 3)*.

The DNA fragment of 350 pb amplified from allele B contains only one restriction site for Hinf I enzyme in position 266, the other one being abolished following the mutation. This way, following digestion with Hinf I enzyme, there will appear two fragments, one of 266 pb and another of 84 pb, corresponding to genotype BB (Figure 1). In case of the heterozygote genotype AB, following digestion there will appear all four types of fragments visualized as three strips of 266 pb, 132/134 pb and 84 pb *(fig. 3)*.

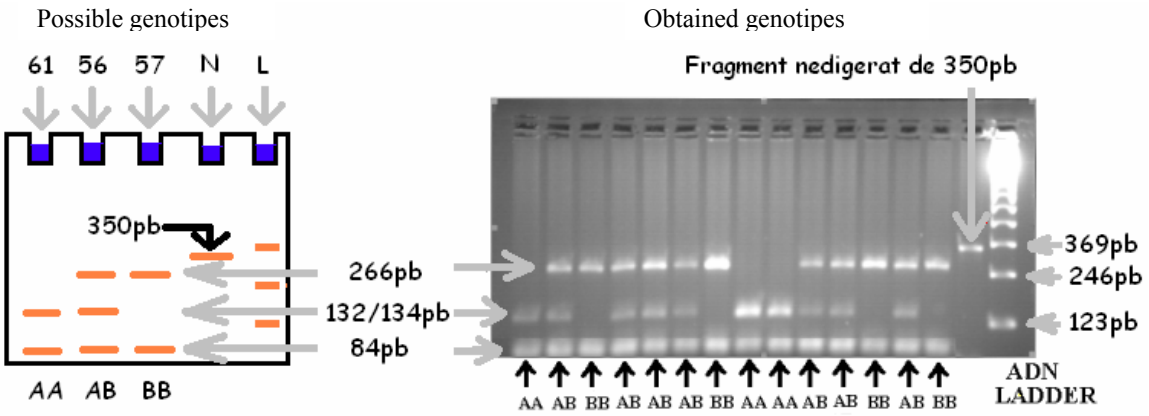

Fig. 3 Electrophoresis profile highlighting the genotypes obtained for K-casein locus by amplification and digestion of a fragment of 350 pb of the gene (part of exon IV and intron IV), belonging to individuals from Romanian Grey Steppe breed from SCDCB Dancu, Iaşi

For the population under study, we identified all three genotypes from K-casein locus estimated by this protocol (AA, AB, BB).

The identification of alleles A and B of β-lactoglobulin was possible by the amplification of a fragment of 262 pb, located between exon IV and intron IV, using specific primers (BGLP3, BGLP4).

The DNA fragment of 262 pb amplified from allele A contains one restriction site for Hae III enzyme in position 109, resulting two types of fragments of 153 pb, and 109 pb, after the digestion with this enzyme. They correspond to genotype AA of this locus *(fig. 4)*.

The DNA fragment of 262 pb amplified from allele B contains two restriction sites for Hae III enzyme, one in position 109 and another in position 188, appeared following the mutation (Figure 2). This way, following digestion with Hae III enzyme, there will appear three fragments of 109 pb, 79 pb and 74 pb, corresponding to genotype BB. Due to an almost similar size and the impossibility for their separation in the agarose gel, the two fragments of 79 pb and 74 pb migrated together and they appeared under the form of one single strip containing a mixture of these two fragments *(fig. 4)*.

In case of the heterozygote genotype AB, following digestion there will appear all four types of fragments visualized as three strips of 153 pb, 109 pb and 74/79 pb *(fig. 4)*.

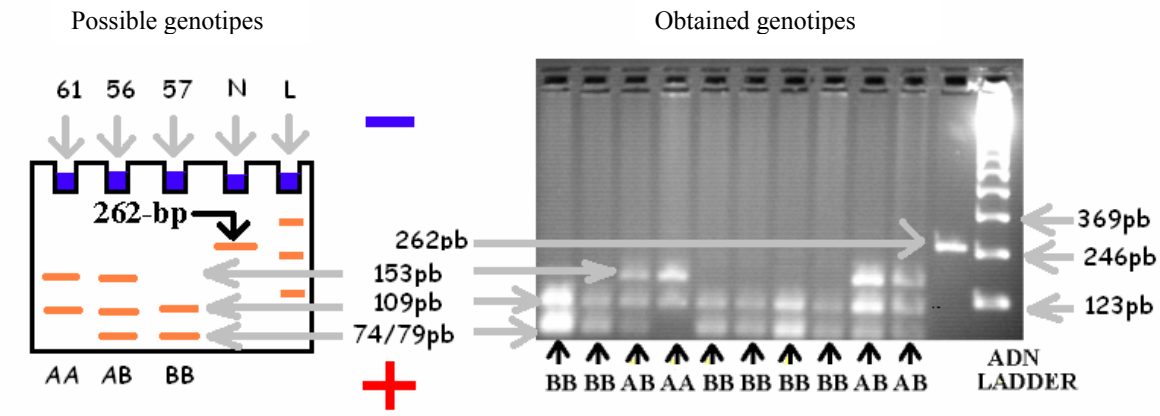

Fig. 4 Electrophoresis profile highlighting the genotypes obtained for β-lactoglobulin locus by amplification and digestion of a fragment of 262 pb of the gene (part of exon IV and intron IV), belonging to individuals from Romanian Grey Steppe breed from SCDCB Dancu, Iaşi

For the population under study, we identified all three genotypes from β-lactoglobulin locus expected by this protocol (AA, AB, BB).

Study of polymorphisms of milk proteins for the Romanian Grey Steppe breed by means of the isoelectric focalization technique (IEF).

Work methods:

The milk samples were collected individually in 15 ml Falcon tubes, transported at 4^0 C and then frozen at -20^0C until tests were run.

Defrosting occurred slowly at room temperature and, subsequently, samples were centrifuged at 8.000 rotations/minute, for 5 minutes for milk separation. They were stored for 30 minutes at 4 degrees for fat solidification and then it was removed from each tube by means of a spatula.

For an optimal protein concentration, samples were diluted with a urea and β-mercaptoethanol solution.

Samples were migrated in a polyacrylamide gel with 4% concentration. After migration, the gel was immersed in a solution 10% of trichloroacetic acid.

Colouring occurred for 2 hours by means of a solution 0.025% Coomassie Brilliant Blue R-250 in 40% ethanol and 7% glacial acetic acid.

In *figure 5* we may see the alleles identifies for the six loci codifying the six types of major proteins of milk ***(α S1-cz; β-cz; K-cz; β-lg; α-la; α S2-cz).***

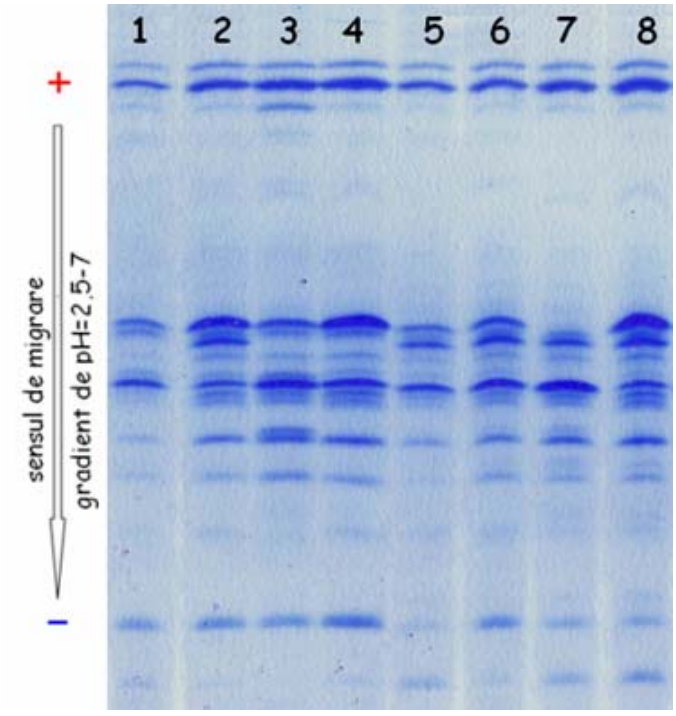

Fig. 5 IEF profile belonging to some individuals from Romanian Grey Steppe breed from SCDCD Dancu highlighting alleles of milk major proteins

The genetic structure for polymorph systems of milk proteins *alpha-casein S_1 (αS1-cz), beta-casein (β-cz), kappa-casein (K-cz), beta-lactoglobulin (β-lg), alpha-lactalbumin (α-la) and alpha-casein S_2 (αS2-cz)* is presented in *table 4.*

Table 4

Genetic polymorphism of milk proteins for the Romanian Grey Steppe breed from S.C.D.C.B. Dancu Iaşi

Registration no.	***α S1-cz***	***β-cz***	***K-cz***	***β-lg***	***α-la***	***α S2-cz***
9991	BI^{RV}	A_1A_2	AB	AB	BB	AA
9993	BB	A_1A_1	BB	AB	BB	AA
9983	BB	A_1A_2	AB	AB	BB	AA
9988	BB	A_1A_2	BB	AA	BB	AA
0004	CI^{RV}	A_2A_2	AA	AB	BB	AA
9985	BC	A_1A_2	AB	AB	BB	AA
9990	BB	A_1A_2	BB	AA	BB	AA
9998	BC	A_2A_2	AB	AB	BB	AA
9723	BB	A_1A_1	BB	AB	BB	AA
9986	BC	A_2A_2	AB	AB	BB	AA
Genotype frequency	$BB = 0.5$ $BC = 0.3$ $CI^{RV} = 0.1$ $BI^{RV} = 0.1$	$A_1A_1 = 0.2$ $A_1A_2 = 0.5$ $A_2A_2 = 0.3$	$AA = 0.209$ $AB = 0.416$ $BB = 0.375$	$AA = 0.292$ $AB = 0.500$ $BB = 0.208$	$BB = 1$	$AA = 1$
Allele frequency	$p_B = 0.7$ $q_C = 0.2$ $r_{IRV} = 0.1$	$p_{A1} = 0.45$ $q_{A2} = 0.55$	$p_{A1} = 0.417$ $q_{A2} = 0.583$	$p_{A1} = 0.542$ $q_{A2} = 0.458$	$P_B = 1$	$p_A = 1$

Casein α_{s1}, in our case α_{s1}-Cn B, is more frequently met, as the specialized literature mentions with a frequency higher than 0.7. For yaks, a high frequency is registered by α_{s1}-Cn C, this being higher than 0.6. Also for yaks, a quite high frequency (above 0.3) is registered by a quite rare variant, α_{s1}-Cn E. Variant α_{s1}-Cn A has not been found so far but in Holstein breed (Aschaffenburg 1968; Gonyon et al., 1987; Ng - Kwai - Hang et al., 1984) and the Red Danish breed (Thymann and Larsen, 1965).

Variant α_{s1}-Cn D, discovered in the Flemish breed (Grosclaude et al., 1966), has a very low frequency (about 0.01), being also found in some French and Italian breeds.

Casein α_{s2} – is monomorphous for Romanian Grey Steppe breed, as it appears at all bovine breeds studied so far. In 1987, Grosclaude found a polymorphism (variant α_{s2}-Cn D) in the breeds Montbéliarde and Vosgienne. In 1981, Mahe highlighted the variants α_{s2}-Cn B and α_{s2}-Cn C for yaks, the two variants having frequencies of 0.1 – 0.2.

Casein β – has the two universal variants β-Cn A_1 and β-Cn A_2 found out at bovines and zebu. Variant β-Cn A_2 is the most frequently met in all breeds studied so far. Variant β-Cn A_1 has a higher frequency in the breeds originating in North-West Europe such as Holstein, Ayrshire, Shorthorn (Kiddy, 1968; Li and Graunt, 1972; Grosclaude and Mahe, 1984), or breeds related to these. *Variant A_1 has a higher frequency in the improved breeds of members of the Bovidae family.*

The higher frequency of allele A_2 has a special significance since this allele is the ancestral one from which all the others derived phylogenetically.

Variant β-Cn A_3 has a much reduced frequency being discovered in the breeds from North-West Europe such as Holstein and Ayrshire breeds (Arave, 1967; Aschaffenburg, 1968; Li and Graunt, 1972), and in some French autochthonous breeds from Normandy.

Variant β-Cn B is also universally encountered in the bovine and zebu breeds with a much reduced frequency. Only in Jersey breed, its frequency is higher reaching 0.4 (Aschaffenburg, 1968, Kiddy et al., 1968; McLean et al., 1984).

Variant β-Cn C has a very low frequency in most European breeds and in those from other continents (Grosclaude, 1974).

They have also discovered a series of very rare variants in different countries such as: β-Cn E in Italy (Voglino, 1972), β-Cn B^2 in New Zealand (Creamer and Richardson, 1975), β-Cn A^4 in Japan (Abe et al., 1975), Mongolic β-Cn A^3, in Mongolia (Grosclaude et al., 1982).

Casein kappa (K Cz). All researches have undoubtedly showed the favorable influence of variant k-Cn B on milk quality, cheese output and quality. Consequently, in the study of bovine lactoproteins, most researches focused on the determination of the frequency of kappa-casein alleles at different breeds and the possibility of "limited" promotion by selection of kappa casein B.

Variants k-Cn A and k-Cn B are universally discovered at bovines and zebu. In recent years, 3 more variants have been identified: k-Cn C, k-Cn D and k-Cn E, all having frequencies lower than 0.1 and being identified only in some local breeds.

Variant k-Cn A has a higher average frequency for most breeds. Thus, in Holstein breed raised in different countries, the frequency of k-Cn A ranges between 0.6 – 0.85.

Variant k-Cn B has a higher frequency in the breeds from Brună group, of different origins raging between 0.4 and 0.6. For Jersey breed, the frequency of k-Cn B is also high (over 0.6). The higher frequency of k-Cn B in these breeds is positively correlated to a high percentage of protein from milk and a higher output in the cheese yield.

The failure to promote k-Cn B by selection triggers in time a reduction of its frequency.

In the crossbreeds of different breeds, the frequency of k-Cn B is intermediate between the frequencies of pure breeds showing the strong influence of crossbreeding in the transmission of the wanted type of kappa-casein.

α-lactalbumin. Variants α-La A and α-La B, apparently exist in most zebu populations (Aschaffenburg, 1968) but we may encounter only variant α-La B in almost all breeds of bovines. Variant α-La A, encountered in zebu, is less rare in the countries from Central and Meridional Europe being discovered in 11 Italian breeds and some Russian and Romanian local ones (according to Mariani and Russo, 1977).

For banteng, they encountered a very rare variant α-La C having the absolute frequency (1.0) for this breed (Bell et al., 1981).

β-lactoglobulin. Two variants, β-Lg A and β-Lg B, are universally encountered at bovines and zebu. The distribution of the two variants in most breeds is quite balanced.

Variant β-Lg C is specific only for Jersey breed (Bell, 1962; McLean et al., 1984) and β-Lg D, discovered in Montbéliarde breed (Graosclaude et al., 1966), but later discovered in other European breeds too, seems to be specific only for the breeds with better aptitudes for meat. Both variants have very low frequencies below 0.1.

Though in phylogeny, variant B is the one from which all the others alleles derived for this locus, the higher frequency of allele A shows a certain work of amelioration carried out on the breed so as to improve the milk quantity.

DETERMINATION OF THE BLOOD GROUPS AND ESTABLISHING THE INDIVIDUALITY OF THE INDIVIDUALS UNDER STUDY

Blood groups were determined by means of the hemolytic tests.

The highlighting of antigen presence is based on the antigen-antibody reaction.

The study of the blood groups in animals has recently known a special development due to the numerous theoretical and practical applications in the raising and improvement of animals. The basic element in the practical application of the blood groups for animals consists in the fact that the blood groups are hereditary and they stay constant throughout the animal's life and, like any other individual, an animal cannot have an antigen in its blood if it did not inherit it from one of its parents.

In current practice, the use of blood groups has applicability in several fields such as: precise *establishing of animal's identity, establishing origin or the paternity test, identification of cases of monozygotic twins, precocious identification of the freemartinism cases, explaining the hemolytic disease, establishing a correlation between a certain type of blood group and certain morpho-physiological and productive particularities.*

From the theoretical viewpoint, blood groups are used to analyse filiation among breeds and populations, establishing the homozygosis or heterozygosis level within a population as well as in the studies of ecologic genetics. All these constitute research domains that gave a maximal certitude coefficient due to the blood groups.

In the members of the Bovidae family, we researched 12 blood groups (A, B, C, F, J, L, M, S, Z, R'S', T', N').

In our researches, by means of the hemolytic tests for the 10 individuals under study belonging to the Grey Steppe breed, we found out 8 blood systems: A, B, C, F, J, L, S, Z. We must say that the livestock is heterogeneous and small since we most frequently encountered *antigens H' (on average 93.0 %), F (87.0 %) and W (73.0 %) and with a smaller frequency antigens* J_2' *(2.0%),* P_2 *(4.0%), B''(6.0%), D' (8.0%),* J_2, T_1, *B', P', G' (9.0 %).*

Bucătaru N. (1996), in his researches made on populations of Simmental crossbred with the Grey Steppe, shows that once with the increase of the participation share of Simmental breed one may notice an increase of frequency for antigens G_3, I_1, P_1, Q, T_1, J_2, B', B'', G'', W, L', F, J and a decrease of frequency for antigens A_2, G_2, I, Q', X_1, X_2.

Knowing the immunogenetic particularities of breeds is absolutely necessary and important in maintaining their genofund. In F.A.O., they give a special attention to the preservation and maintaining of genofunds of diverse animals species. Consequently, in the case of the Grey Steppe we must also highlight the differences appearing between the pure bred and its crossbreds with Brună and Simmental breeds.

MAKING THE KARYOTYPE OF THE INDIVIDUALS UNDER STUDY

In the last decades, both at world level and in our country, a major desideratum regarding the raising of farm animals has been the maintaining of genetic health of livestocks. The identification of the bearers of alterations of the genetic material and recommending the necessary measures to prevent the dissemination of these hereditary defects in the descendant populations is the objective of genetic prophylaxis which does not aim at treating genetic diseases but preventing them. The need of genetic prophylaxis measures is imperative for all breeds of members of the Bovidae family raised in our country. Starting from these premises, we consider that our study will contribute, on one hand, to the underlining of the idea of danger represented by the chromosomal anomalies and, on the other hand, to the preservation of a genetically healthy Romanian Grey Steppe livestock.

This year, we have established a 10 cow group belonging to the Grey Steppe breed and it was cytogenetically investigated.

After having filling in the origin data, we aseptically harvested blood samples necessary to make the karyotype.

The chromosomal preparations were made by the technique of microculture from integral blood, with some modifications required by the destination of preparations. The white cells of peripheral blood were cultivated for 72 hours on a culture environment MEM Eagle with Earle and $NaHCO_3$ (Sigma) salts supplemented by 15% calf fetal serum (Sigma), solution N-16 (IC) and phytohemaglutinine M(Difco) as mitogen. During the last 2 hours of culture, we added ethidium bromide (10µg/ml) to inhibit the contractions of chromosomal structures. One hour before the opening of cultures, we added a solution of colquicine 0.004% to block mitoses in metaphase, then we started the following processing stage according to the conventional protocol, namely hypotonization in 0.075M KCl, fixations repeated in methanol/glacial acetic acid (3:1) and display on cold slides. Colouring was made by means of Giemsa 10%. For each individual, we made two cultures. Mitotic preparations were examined under Nikon-Optiphot 2 microscope, in direct light. Their quantity allowed us to analyse 100 metaphases for each individual.

The entire chromosomal analysis process, from the identification of mitotic figures up to the qualitative and quantitative evaluation, was executed by means of specific equipment for image analysis. In the current experimental conditions supported by the computerized analysis of images, the chromosomal preparations were analysed in microscopic field and the best mitotic figures were photographed by a video camera mounted on Nikon microscope and processed in specific files of PCX type.

The cytogenetic examination effectuated on 10 Romanian Grey Steppe cows confirmed the existence of a normal chromosomal complement 2n=60, XX.

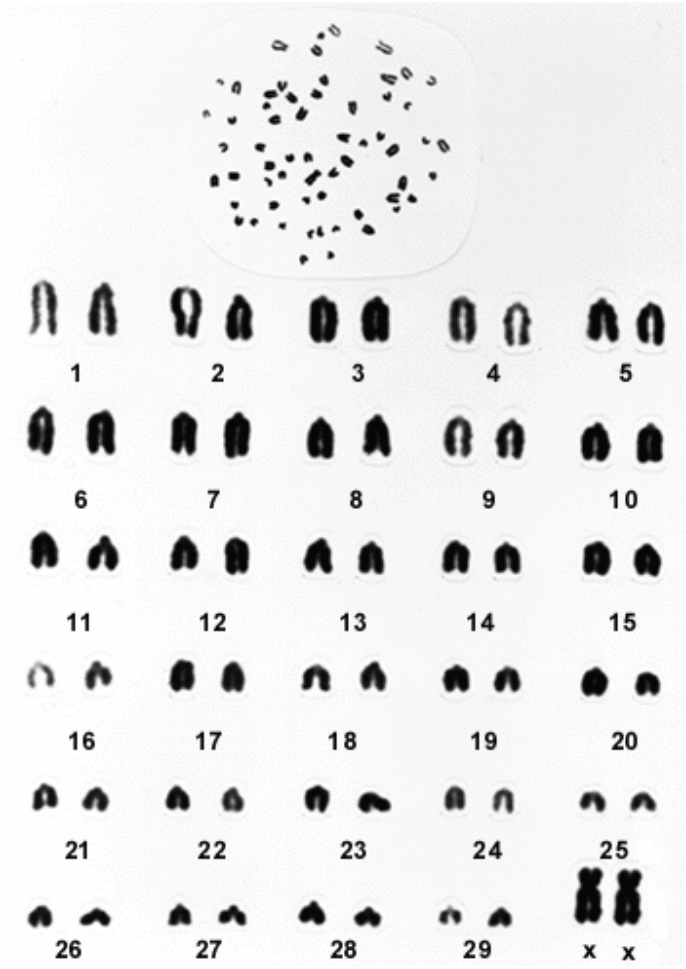

Fig. 6 Normal karyotype, 60,XX for females from Romanian Grey Steppe breed

The goals and activities for 2009 year have been fully acomplished

PROJECT MANAGER,

A/P Creangă Ştefil, Ph.D.