Phenotypic characterization of the Romanian Grey Steppe breed

Objectives:

1. ***Establishing the group of animals under analysis;***
2. ***Specialized documentation, elaboration of applicative biometrical programmes;***
3. ***Making up the database, completing the experimental protocols;***
4. ***Phenotypic characterization of the Romanian Grey Steppe breed;***
5. ***Estimation of the statistic indicators and genetic parameters;***
6. ***Establishing protocols to determine the molecular markers (genetic and biochemical)***

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

The activities carried out within the research project for 2008 stage aimed at:

- Identification and registration of the individuals and groups under study;
- Measurements and specific determinations;
- Elaboration of experimental models, specialized documentation for the adoption of the biometrical models;
- Specific registrations to make up the database;
- Elaboration of the specialized documentation to process the registered database;
- Analysis of the morphological and production characters *(determining the body development based on measurements and appreciation of conformation by linear description, control or the milk yield, data management in terms of origin, mounts, calving, obtained products, entries and exits from the monitored nucleus);*
- Analysis of the production features;
- Estimation of the average values and variability, calculation of the significance tests;
- Estimation of heritability, repeatability of phenotypic, genotypic and environmental correlations;
- Elaboration of experimental models to establish the individuality of animals;
- Establishing the protocols to determine the molecular markers (genetic and biochemical).

The importance of protection and preservation of the animal genetic resources in all the countries of the world consists in the permanent preoccupation of FAO (Food and Agriculture Organization of the United Nations) by annual meetings with the designated specialists from every country and numerous publications elaborated periodically through **IDAD** (Informative for Domestic Animal Diversity) such as: the red list for worldwide threatened animals and plants; worldwide strategy and management of genetic resources of the farm animals; the main Guidelines for the development of the management plans of genetic resources at national level. Taking into account that Romania signed in 1992 the ***UNO Convention for biological biodiversity*** and the harmonization of the national legislation with the international one, we need to revise the strategy for the preservation of genetic resources at national level.

Agrobiodiversity is an essential element of world biodiversity. More than 75% from the world agricultural production comes from 25 domestic vegetal and animals species. The management and modifications of the Genetic Resources within these species are indispensable and ensure food security. ***Identification, obtaining and subsequent development of the vegetal and animal genetic material*** leading to a high productivity and a good adaptation to local conditions ***are world priorities*** and have led to an unprecedented movement of the genetic material for agricultural use.

In this stage, it is necessary to run a ***census*** of the *animal genetic resources*. The number of domestic animal breeds exploited at present is about 40, out of which 14 breeds ensure 90 % from total animal production. Nine of these 14 breeds (bovines, horses, swine, sheep, goats, buffaloes, poultry and turkey hens), representing about 4,000 breeds from the entire world, make up the largest part from the total number of mammals known on earth. Following a world inquiry on 28 domestic species, they found 3,882 breeds, out of which **880 do not have a precise animal population**, they are ***in danger*** and ***their genetic type may disappear. .***

Food and Agriculture Organization of the United Nation (FAO) has a long history of participation in the world management of the animal genetic resources and accepted the role of a leader and coordinator of the actions deployed for the monitoring of the genetic resources of domestic animals. F.A.O. launched in 1995 an extended Programme for the management of the genetic resources of farm animals for whoever supplies a mechanism for international communication and cooperation related to the management of the animal genetic resources. The technical elements of FAO's world Programme lays accent on the identification, description, development, use and supervision of the animal genetic resources, the preservation of the unique and threatened resources, formation and participation of population in the management of the animal genetic resources and improvement of communication by dialogue and international relations for the management of the animal genetic resources.

The key points for the application of the inventory of national animal genetic resources shall be made for every breed, as follows:

1. identification of different breeds;
2. description of every breed;
3. evaluation of the number of animals from every breed and evolution of populations in time;
4. comparative characterization of the main production characters and adaptation to the main production conditions;
5. evaluation of world importance of national breeds;
6. supervision of the future modification within the breed.

The world data bank – F.A.O. for animal genetic animal resources is at Rome as well as the central storage system of information about animal genetic resources. It contains information about 4,000 breeds of mammals and poultry.

The information contained in the world data Bank from F.A.O. and the forms for information support FAO/UNEP(1995) and DAD-IS are available to the persons designated to run the national inventories of the animal genetic resources they being thus encouraged to use the forms and lists of description from FAO, so as to take advantage of the organization's experience in this field and to facilitate the entry of the future national information in the world data bank. Moreover, the DAD-IS breed bank is available for direct use at national level eliminating the need to create a new database.

The information and communication system of FAO, DAD–IS, which is developed and kept by FAO for the countries managing animal genetic resources, helps the national institutions responsible for the data synthesis of every country and the information distribution for the study of the national, regional and international priorities.

RESULTS OBTAINED

At present, it is unanimously appreciated that modern society is facing numerous essential issues for mankind's future appearing as a result of the disproportion between an unprecedented demographic explosion and the natural resources which in some places are diminishing and rapidly degrading following faulty exploitation. Due to these facts, *the issue of environment preservation* has become necessary, an issue present today on the agenda of numerous governments and international bodies.

Romanian Grey Steppe breed, on the verge of extinction, has been included in the preservation programme of animal genetic resources and it is being raised at the Research and Development Station for Bovine Growing – Dancu Iaşi.

We do not have recent data about the Romanian grey Steppe breed in terms of morphoproductive characters and the current genetic value, a reason why our team has started studying the nucleus exiting at Dancu Station.

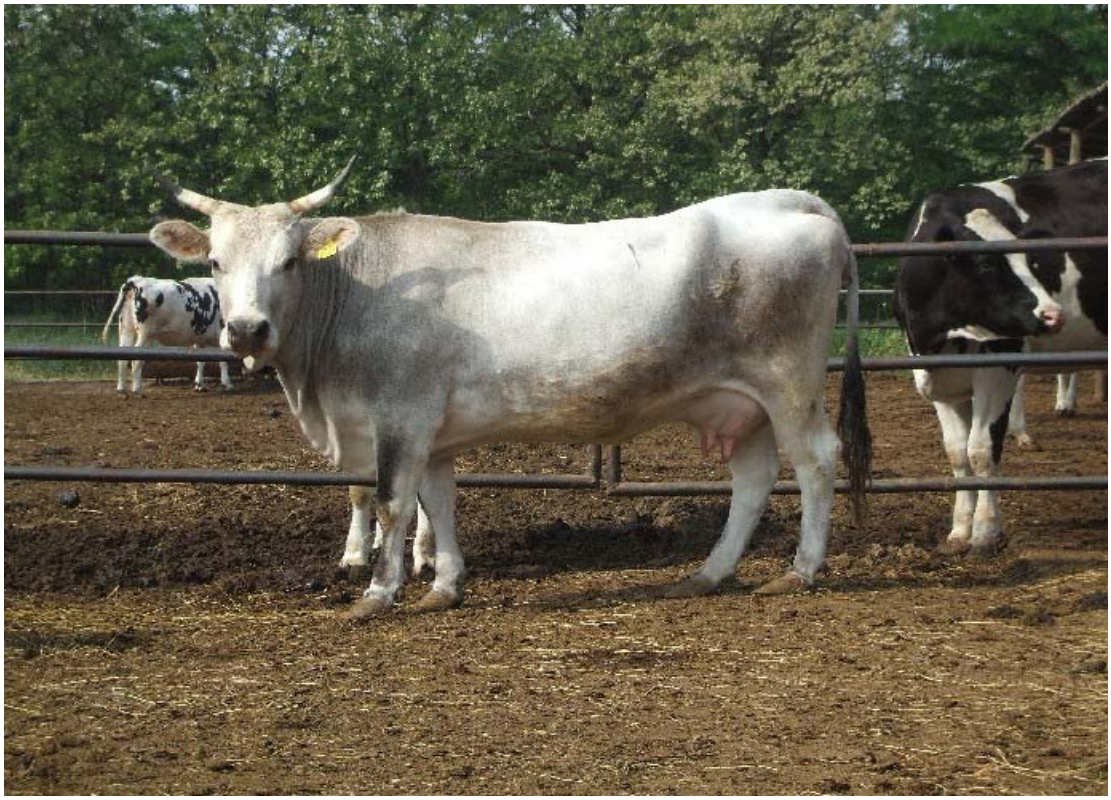

Fig. 1. Romanian Grey Steppe breed at S.C.D.C.B. Dancu Iaşi

Romanian Grey Steppe *(fig.1)* is an unimproved breed coming from the original form of Bos taurus primigenius. The formation of the breed is tightly related to the natural environment where the cow keeper contributed very little.

In our country, up to the first half of the past century, the breed was preponderant but subsequently its share seriously decreased. At present, it may be found sporadically as crossbreds with a different degree of absorption in the Danube Delta, Moldavia and a compact population of 35 heads at S.C.D.C.B. Dancu, Iaşi county and SC. Trei Brazi, Neamț county. Several varieties are known that were called after their formation area: *Moldavian, Transylvanian, Ialomita and Dobrudjan.* The Transylvanian variety is characterized from the morphologic viewpoint by a large body development followed by the Moldavian variety situated among the medium size bovines also having the largest spread in the Moldavian area in the past.

MATERIAL AND METHOD

Researches were effectuated on 30 Romanian Grey Steppe cows on which we studied:

- the indices of milk yield by successive lactations (the first eight lactations);
- the indices of body development for the adult animals;
- intrapopulational genetic structure;
- heritability, repeatability, phenotypic and genetic correlations among the main morphoproductive characters.

The data were taken from observations and direct determinations in the raising area of the breed as well as from primary data bank of S.C.D.C.B. Dancu - Iaşi and UARZ Iaşi.

All data were statistically processed by a biometric model and a programme elaborated within the Animal genetics and Bovine growing technology departments (S.A.V.C., Statistics, Variance and Covariance Analysis etc, *A/P Creangă Ştefil, Ph.D., A/P Vasile MACIUC, Ph.D., 2000-2003*).

RESULTS OBTAINED AND DISCUSSIONS

In *table 1*, we give the average values and variability of milk yield indices by successive lactations for the Romanian Grey Steppe breed. As one may see, the duration of total lactation is also the duration of normal lactation since the 305 day lactation period is not exceeded. The milk quality per lactation ranged between 1589.64 kg (1st lactation) and 2535.43 kg in the 5th lactation. Starting from the 6th lactation, the milk quantity decreases and in the 8th lactation it reaches 1078.5 kg.

In the 1st lactation, 62.69 % from the maximal lactation was attained, a value highlighting the tardiness of Romanian Grey Steppe breed in terms of milk yield.

The variability of the milk quantitative production is very strong, the values of the standard deviation ranging between s = 544.10 kg in the 1st lactation and 1185.89 kg 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 improvement by retaining and multiplying the valuable genotypes. We must mention that for the nucleus under study there were individuals with a maximum yield of 4080 kg milk or 3080 kg per lactation.

In the genetic structure of the livestock under study, we identified three groups of paternal semi-sisters (*table 2*) with yields of 1548.22 kg (code 79009) and 1752.33 kg (code 79005), quite small values in terms of milk yield.

However, we may notice a good body development of the genetic groups with values of body weight between 549.38 kg (87027) and 626.67 kg (code 79005). These data are favorable for the selection of the nucleus under study in order to improve the meat yield of Romanian Grey Steppe breed.

Table 1

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

Specification	Sample statistics	Total lactation				Normal lactation				
		Duration in days	Milk kg	% fat	Kg fat	Duration in days	Milk Kg	% fat	Kg fat	Gestation period
1st lactation	n	30	30	30	30	30	30	30	30	30
	$\overline{X}$	259.80	1589.64	4.64	68.94	259.80	1589.64	4.64	68.94	281.89
	±s $\bar{x}$	14.04	112.51	0.09	5.03	11.18	102.82	0.09	4.67	1.07
	s	74.33	595.35	0.49	26.62	59.19	544.10	0.49	24.74	5.70
	V%	28.68	36.69	11.24	39.18	23.74	36.43	11.21	37.48	2.02
	Min	90	360	3.40	15.00	90	360	3.40	15.00	269
	Max	450	2612	5.30	110.00	305	2612	5.30	107.00	293
2nd lactation	n	27	27	27	27	27	27	27	27	27
	$\overline{X}$	254.26	1699,96	4,65	67,04	254.26	1699.96	4.65	67.04	277.26
	±s $\bar{x}$	14.45	147.15	0.09	5.09	14.45	147.15	0.09	5,09	5,42
	s	62.29	705.71	0.45	24.42	62.29	705.71	0.45	24.42	25.99
	V%	27.25	41.58	9.88	33.43	27.25	41.58	9.88	33.43	9.37
	Min	32	198	3.70	10.00	32	198	3.70	10.00	161
	Max	369	3565	5.40	111.00	369	3565	5.40	111.00	296
3rd lactation	n	20	20	20	20	20	20	20	20	20
	$\overline{X}$	254.80	2092.80	4.51	93.00	254.80	2092.80	4.51	93.00	284.50
	±s $\bar{x}$	15.39	215.08	0.12	8.76	15.39	215.08	0.12	8.76	1.35
	s	59.61	833.01	0.49	33.95	59.61	833.01	0.49	33.95	5.08
	V%	23.39	39.80	10.85	36.50	23.39	39.80	10.85	36.50	1.78
	Min	116	434	3.50	22.00	116	434	3.50	22.00	276
	Max	345	4080	5.30	144.00	345	4080	5.30	144.00	292
4th lactation	n	15	15	15	15	15	15	15	15	15
	$\overline{X}$	290.50	2082.10	4.62	91.10	290.50	2082.10	4.62	91.10	278.70
	±s $\bar{x}$	22.75	250.46	0.13	9.71	22.75	250.46	0.13	9.71	3.18
	s	71.95	792.03	0.41	30.72	71.95	792.03	0.41	30.72	10.06
	V%	24.77	38.04	8.94	33.72	24.77	38.04	8.94	33.72	3.61
	Min	194	835	4.10	45.00	194	835	4.10	45.00	255
	Max	470	3080	5.30	138.00	470	3080	5.30	138.00	292

Table 1 *(continuation)*

Specification	Sample statistics	Total lactation				Normal lactation				
		Duration in days	Milk kg	% fat	Kg fat	Duration in days	Milk kg	% fat	Kg fat	Gestation period
5th lactation	**n**	8	8	8	8	8	8	8	8	8
	$\overline{X}$	285.43	2535.43	4.73	119.92	285.43	2535.43	4.73	119.92	282.00
	±s $\overline{x}$	20.76	448.22	0.21	25.25	20.76	448.22	0.21	25.25	3.20
	s	54.93	1185.89	0.57	66.82	54.93	1185.89	0.57	66.82	8.48
	V%	19.24	46.77	12.06	54.01	19.24	46.77	12.06	54.01	3.00
	Min	191	675	3.70	32.00	191	675	3.70	32.00	263
	Max	369	4087	5.30	212.00	369	4087	5.30	212.00	287
6th lactation	**n**	5	5	5	5	5	5	5	5	5
	$\overline{X}$	226.75	1411.00	4.95	69.00	226,75	1411.00	4,95	69.00	281.33
	±s $\overline{x}$	21.04	201.67	0.15	9.28	21.04	201.67	0.15	9.28	1.66
	s	42.08	403.35	0.31	18.56	42.08	403.35	0.31	18.56	2.88
	V%	18.55	28.58	6.28	26.90	18.55	28.58	6.28	26.90	1.01
	Min	175	818	4.60	43.00	175	818	4.60	43.00	282
	Max	278	1705	5.30	87.00	278	1705	5.30	87.00	287
7th lactation	**n**	3	3	3	3	3	3	3	3	3
	$\overline{X}$	298	1519.00	4.66	83	298	1519.00	4.66	83	276
	±s $\overline{x}$	20.75	270.46	0.13	9.71	20.75	270.46	0.13	9.71	3.18
	s	71.95	792.03	0.41	30.72	71.95	792.03	0.41	30.72	10.06
	V%	14.77	18.04	3.94	13.72	14.77	18.04	3.94	13.72	3.61
	Min	254	835	4.10	45.00	254	835	4.10	45.00	250
	Max	350	1980	5.30	138.00	350	1980	5.30	138.00	290
8th lactation	**n**	2	2	2	2	2	2	2	2	2
	$\overline{X}$	227.77	1078.5	5.28	57.44	227.77	1078.5	5.28	57.44	275
	±s $\overline{x}$	10.75	268.46	0.13	9.71	10.75	268.46	0.13	9.71	3.18
	s	71.95	792.03	0.41	30.72	71.95	792.03	0.41	30.72	10.06
	V%	9.77	12.04	2.94	10.72	9.77	12.04	2.94	10.72	3.61
	Min	194	875	5.19	45.00	194	875	5.19	45.00	254
	Max	260	1282	5.30	68.00	260	1282	5.30	68.00	297

The significance of differences was tested for the groups of semi-sisters reaching the conclusion that there are no great differences in terms of milk yield, the Fisher test indicating 0.6431 (F) < F0.05 $_{(4\ ;\ 22)}$. As for waist, the differences are insignificant 1.1503 (F) < F0.05 $_{(4;\ 22)}$. We noticed significant differences for the body weight presented in table 3.

Table 2

Average values and variability of the main morphoproductive features, by genetic groups, for the Romanian Grey Steppe breed

Specification	UM	Code 79005							Code 79009							Code 87027						
		n	$\overline{X}$	±s $\overline{x}$	s	V%	Min	Max	n	$\overline{X}$	±s $\overline{x}$	s	V%	Min	Max	n	$\overline{X}$	±s $\overline{x}$	s	V%	Min	Max
Gestation period	**days**	3	284.67	1.76	3.05	1.07	282	288	9	283.78	1.50	4.52	1,59	278	291	10	278.30	1.51	4.78	1.72	269	283
Normal lactation length	**days**	3	281.33	13.28	23.00	8.17	258	304	9	247.89	12.42	67.27	27,13	90	305	9	270.33	17.72	53.18	19.67	150	305
Mil quantity	**kg**	3	1752.33	269.80	467.31	26.66	1213	2037	9	1548.22	226.46	679.40	43.88	360	2296	9	1558.11	138.47	415.42	26.66	940	1989
Fat content	**%**	3	4.70	0.20	0.34	7.37	4.50	5.10	9	4.74	0.11	0.33	7.07	4.30	5.30	9	3.98	0.09	0.27	6.85	3.40	4.20
Fat quantity	**kg**	3	81.10	10.05	17.40	21.46	61.00	91.30	9	73.00	10.38	31.14	42.65	15	107	8	62.44	5.98	17.95	28.74	35	84
Withers height	**cm**	3	122.00	3.00	5.19	4.25	119	128	9	123.17	0.74	1.83	1,49	120	125	8	121.75	1.26	3.57	2.93	115	127
Thorax perimeter	**cm**	3	198.33	5.20	9.01	4.54	189	207	9	193.33	1.90	4.67	2.41	187	199	8	189.75	3.00	8.49	4.47	176	201
Body weight	**kg**	3	626.67	49.10	85.04	13.57	540	710	9	580.50	17.68	43.33	7.46	520	630	9	549.38	26.10	73.84	13.44	435	650

Table 3

Significance of differences for body weight among the semi-sister groups

Fisher Test: 12.0429 (F) > F0.001 (4 ; 22) 6.81 * highly significant**								
Tukey Test:								
Character 1	***Character 2***	***Aver. diff.***	***Q1***	***Q2***	***W1***	***W2***	***Significance***	***Threshold***
tg87027	tg79005	77.29					Insignificant	
tg87027	tg79008	117.50					significant	for threshold 0.05
tg87027	tg79009	31.13					Insignificant	
tg87027	tg86002	130.63					significant	for threshold 0.05
tg86002	tg79005	53.33					Insignificant	
tg86002	tg79008	248.13					significant	for threshold 0.01
tg86002	tg79009	99.50					Insignificant	
tg79009	tg79005	46.17					Insignificant	
tg79009	tg79008	148.63					significant	for threshold 0.01
tg79008	tg79005	194.79	4.20	5.23	108.99	135.72	significant	for threshold 0.01

In *table 4* we present the average values and variability of body development from whose analysis we may summarize the following:

- The cows from the nucleus under study have the average waist of 122.28 cm and the body weight of 542.86 kg, values highlighting good body massiveness. For this character, we also notice plus variants reaching the weight of 710.00 kg;
- The waist variability is less accentuated, the group under analysis being sufficiently homogenous (s = 3.06 cm, and V % = 2.51). In exchange, body weight presents a high variability with dispersion indices s = 99.38 kg, and V % =18.30;
- The knowledge of the existing variability reserves, of phenotypic and genetic parameters is very important for a cow population, this determining its improvement stage and ensuring the scientific substantiation of the future improvement and genetic preservation programmes.

Table 4

Average values and variability of body development for the Romanian Grey Steppe breed

Specification	UM	n	$\overline{X}$	±s $\bar{x}$	s	V%	Min	Max	% of waist
Withers height	cm	30	122.28	0.57	3.06	2.51	115.00	128.00	100.00
Back height	cm	30	121.66	0.58	3.14	2.58	116.00	129.00	99.49
Croup height	cm	30	125.14	0.61	3,33	2.66	119.00	132.00	102.33
Tail base height	cm	30	126.00	0.58	3.14	2.49	120.00	132.00	103.41
Thorax width	cm	30	70.24	0.71	3.87	5.50	63.00	79.00	57.44
Sternum height	cm	30	51.62	0.74	4.02	7.79	41.00	60.00	42.21
Oblique length of body	cm	30	155.10	1.90	10.23	6.59	132.00	169.00	126.84
Horizontal length of body	cm	30	134.72	1.14	6.16	4.57	121.00	148.00	110.17
Total length of body	cm	30	191.07	4.67	25.19	13.18	78.00	216.00	156.25
Thorax length	cm	30	87.21	0.97	5.25	6.02	77.00	99.00	71.31
Croup length	cm	30	49.66	0.51	2.79	5.62	44.00	59.00	40.15
Head length	cm	30	48.66	0.57	3.09	6.36	36.00	53.00	40.59
Thorax width behind shoulder blades	cm	30	47.00	0.64	3.47	7.39	41.00	53.00	38.43
Breast width	cm	30	41.03	0.54	2.93	7.14	35.00	47.00	33.55
Croup width at hips	cm	30	49.83	0.50	2.70	5.41	43.00	57.00	40.75
Croup width at coxofemural articulation	cm	30	43.76	0.42	2.27	5.20	40.00	51.00	35.78
Croup width at ischia	cm	30	16.90	0.42	2.27	13.45	13.00	22.00	13.82
Head width	cm	30	21.79	0.18	1.01	4.65	20.00	23.00	17.81
Thorax perimeter	cm	30	189.00	2.18	11.75	6.22	158.00	207.00	154.56
Shinbone perimeter	cm	30	18.07	0.15	0.82	4.54	17.00	20.00	14.77
Body weight	kg	30	542.86	18.45	99.38	18.30	390.00	710.00	443.94

In *tables 5-6* and *fig. 2-7,* we present the values of the heritability and repeatability coefficients and the correlations among the main morphoproductive characters.

From the analysis of heritability of the main morphoproductive characters, we may withhold the medium values for the milk quantity and fat (h^2 = 0.30; 0.31), proving a good genetic consolidation of the studied nucleus. The same aspect also results from the analysis of waist heritability and body weight, the phenotypic selection for these characters being efficient. We must mention the milk fat percentage having a high genetic determinism (h^2 = 0.71).

The repeated determination of an individual's performances prolongs the time for more precise knowledge and consequently the time to make a decision related to its withholding or rejection in the selection process. That is why, to benefit from this increased certitude without resorting to repeated measurements in order to definitely establish the animal's value, we may use a genetic parameter giving details about the degree of resemblance of characters' value between measurements, a genetic parameter called *repeatability*. In a broader meaning, repeatability refers to the phenotypic manifestation of the same character in different periods of individual's life.

The repeatability of analysed characters proves the same good genetic consolidation of the Romanian Grey Steppe breed and the possibility of improvement by phenotypic selection. We highlight the milk quantity and fat with medium repeatability R = 0.33-0.34 and the milk fat percentage with high repeatability R = 0.73.

One of the basic problems of the study of quantitative characters' heredity is the knowledge of the degree of interdependence between two characters highlighted by means of the *correlation coefficients.*

As for the phenotypic and genetic correlations among different analysed features, we show different values and significances depending on the analysed characters *(table 6).* Among the numerous pairs of characters under study, the positive and intense phenotypic and genetic correlations between the milk yield and the fat quantity (r_{pg} = 0.98-0.97) drew our attention. The same positive correlations with a medium intensity are noticed between the milk yield and the body development features (r_{pg} = 0.33 – 0.44). The classical correlation between the milk yield and the fat content is negative (-0.21, -0.19). The correlations between the gestation period and some productive features and body development are weak and positive or weak and negative.

Table 5

Heritability (h^2) and repeatability (R) of the main morphoproductive characters for the Romanian Grey Steppe breed

Specification	Heritability	Repeatability
Gestation period	0.15	0.17
Normal lactation duration	0.27	0.29
Milk quantity	0.30	0.33
Fat percentage	0.71	0.73
Fat quantity	0.31	0.34
Withers height	0.37	-
Thorax perimeter	0.33	-
Body weight	0.41	-

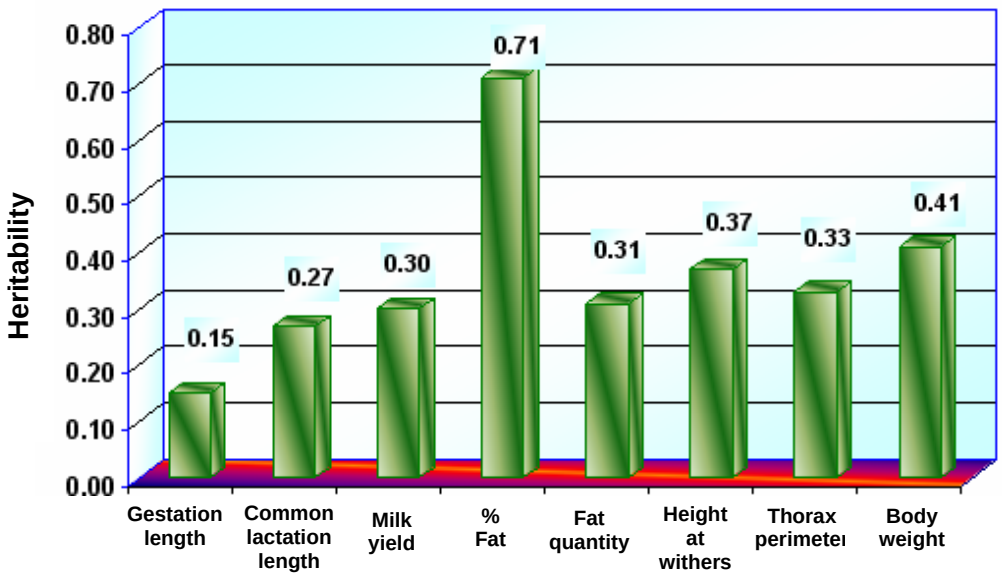

Fig. 2. Heritability coefficient for the main morphoproductive features

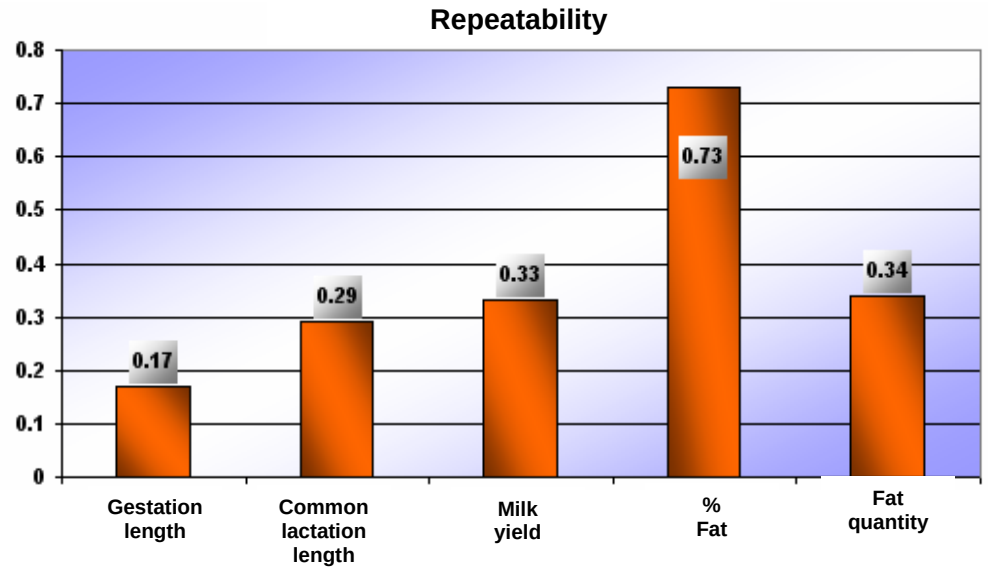

Fig. 3. Repeatability coefficient for the main morphoproductive features

Table 6

Phenotypic (r_p) and genetic (r_g) correlations among the main morphoproductive characters of the Romanian Grey Steppe breed

Correlated features	$r_p \pm s_{rp}$		$r_g \pm s_{rg}$	
Milk quantity and:				
Gestation period	0.18	0.07	0.25	0.04
Lactation length	-0.25	0.03	-0.26	0.05
Fat percentage	-0.21	0.02	-0.19	0.01
Fat quantity	0.98	0.01	0.97	0.03
Waist	0.38	0.06	0.33	0.01
Thorax perimeter	0.39	0.06	0.32	0.01
Body weight	0.44	0.06	0.41	0.01
Gestation period and:				
Lactation length	0.09	0.08	0.13	0.06
Fat percentage	0.07	0.08	0.10	0.07
Fat quantity	0.28	0.07	0.43	0.09
Waist	-0.11	0.01	-0.13	0.05
Thorax perimeter	-0.10	0.07	-0.30	0.04
Body weight	-0.08	0.09	-0.11	0.02

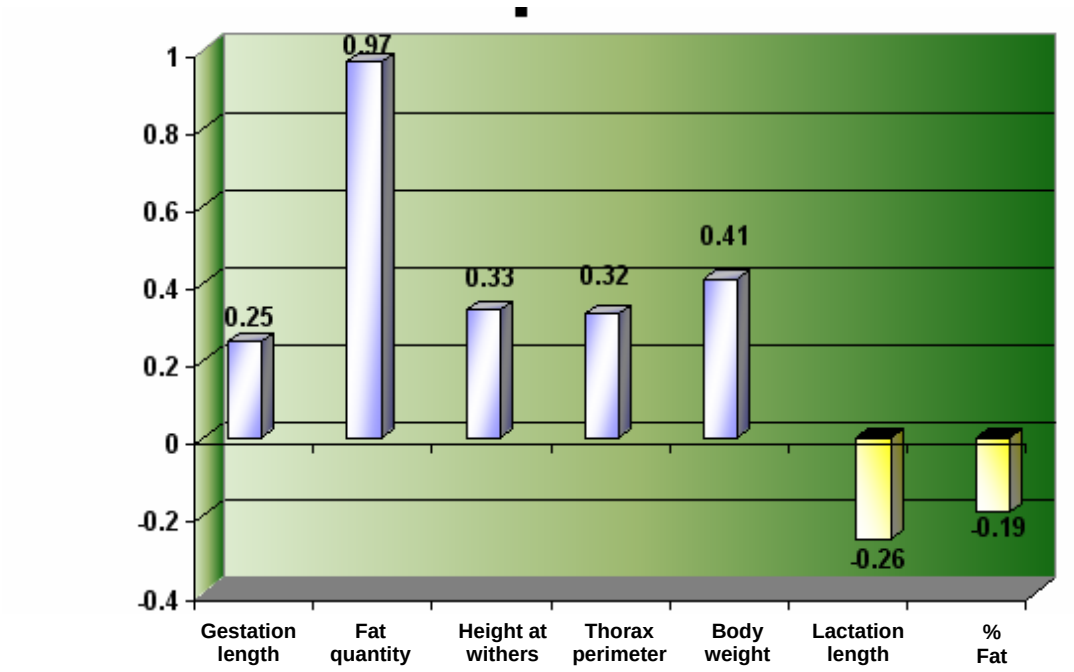

Fig.4. Genetic correlations between the milk quantity and some morphoproductive features

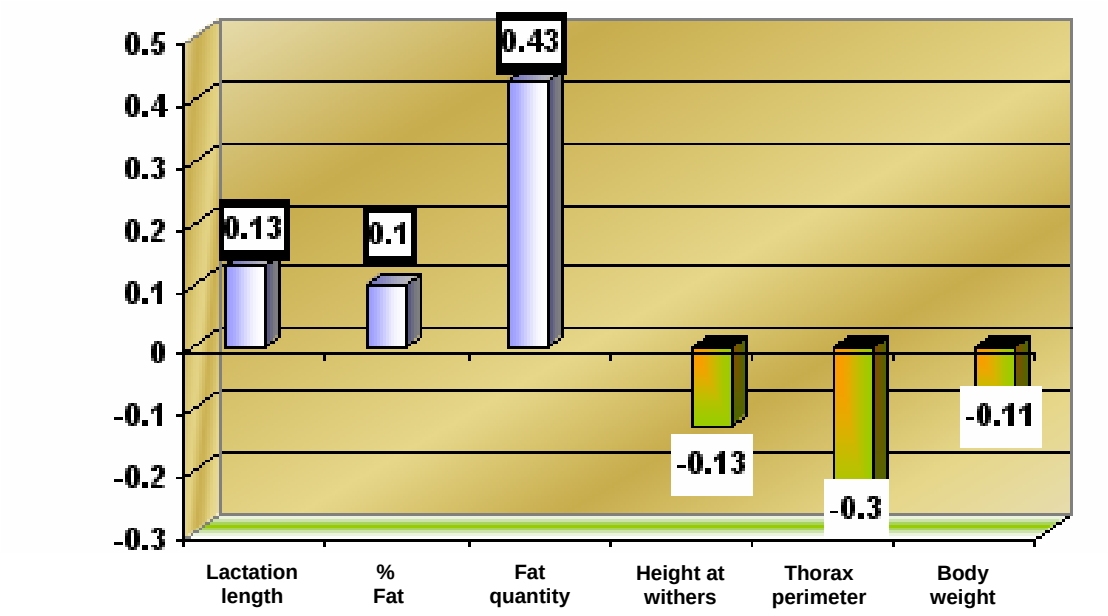

Fig. 5. Genetic correlations between the gestation period and the main morphoproductive features

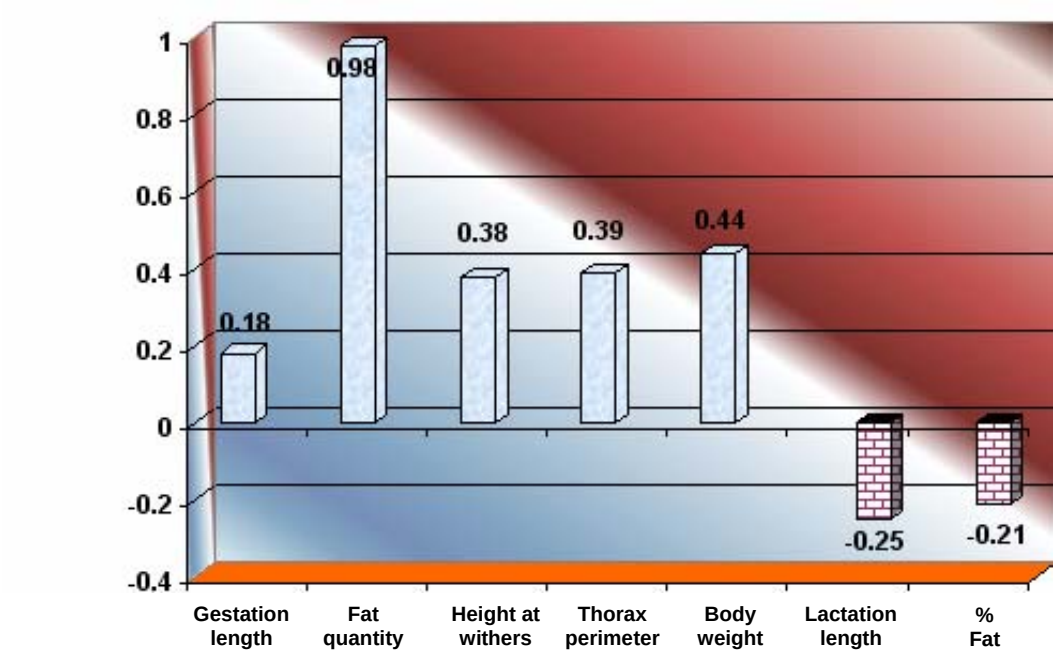

Fig. 6. Phenotypic correlations between the milk quantity and some morphoproductive features

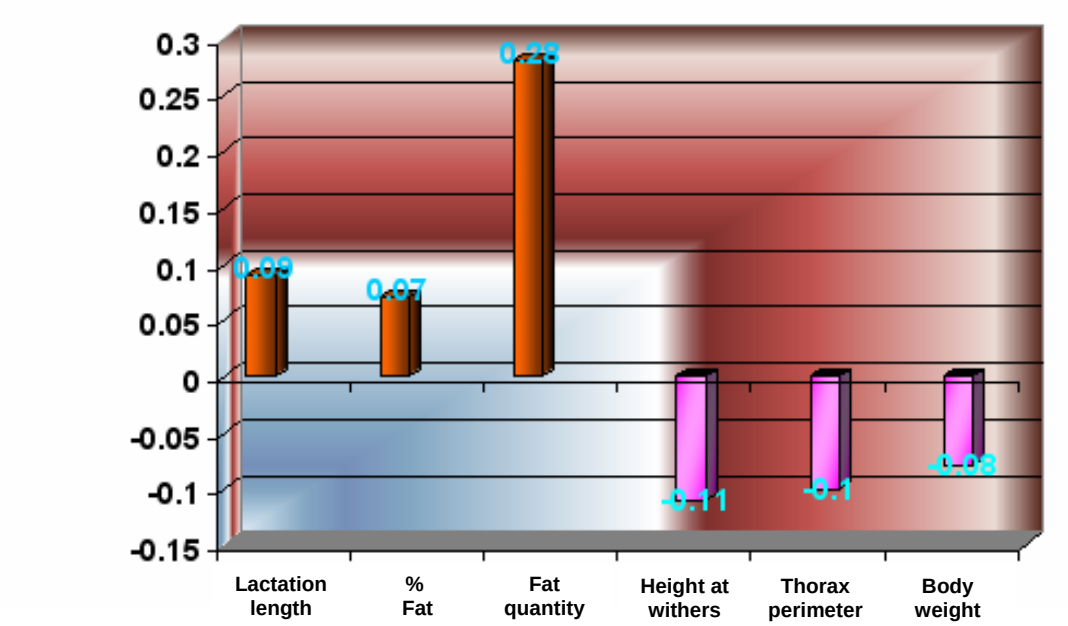

Fig. 7. Phenotypic correlations between the gestation period and the main morphoproductive features

Conclusions

✓ The modern society is facing numerous essential issues for mankind's future appearing as a result of the disproportion between an unprecedented demographic explosion and the natural resources which in some places are diminishing and rapidly degrading following faulty exploitation. Due to these facts, *the issue of environment preservation* has become necessary, an issue present today on the agenda of numerous governments and international bodies;

✓ From the analysis of the morphoproductive indices of the nucleus of Romanian Grey Steppe breed raised at Dancu farm–Iasi results the current genetic value and the possibility for an efficient selection to preserve the existing nuclei. The milk quantity ranged between 1589.64 kg (1^{st} lactation) and 2535.43 kg in the 5^{th} lactation which was also the maximum lactation;

✓ The breeding stock used is not genetically testes and its ascendance is unknown, an aspect influencing the productive level of the population under study;

✓ From the analysis of heritability of the main morphoproductive characters, we may withhold the medium values for the milk quantity and fat (h^2 = 0.30; 0.31), proving a good genetic consolidation of the studied nucleus. The repeatability of analysed characters proves the same good genetic consolidation of the Romanian Grey Steppe breed and the possibility of improvement by phenotypic selection;

✓ Among the numerous pairs of features analyzed, our attention is attracted by the positive and intense genetic and phenotypic correlations between the milk yield and the fat quantity (r_{pg} = 0.98-0.97). The same positive correlations and of medium intensity may be noticed between the milk yield and the body development features r_{pg} = 0.33 – 0.44;

✓ The nucleus of Romanian Grey Steppe breed from Dancu farm represents a valuable genetic fund that must be preserved and meliorated in the sense of the mixed production of meat and milk and developed from the numeric viewpoint so as to avoid the genetic drift and tight consanguinity.

Establishing the work protocols to determine the molecular markers (genetic and biochemical)

Techniques used to identify polymorphisms of milk proteins at the level of genic expression:

Vertical electrophoresis in polyacrylamide gel

This technique was introduced by Rymond and Weintraub in 1959. Its wide scale use started only in 1964 proving to be the most adequate support medium for analytical electrophoretic separations. Since then it has been successfully used for the separation of several protean species, the separation of DNA fragments and sequencing.

This type of electrophoresis uses as a migration support the polyacrylamide gels and migration takes place in vertical system. They are obtained by polymerization of acrylamide and metilenbisacrylamide that is induced by adding some polymerization agents in the solution of these monomers.

Polymerization must tae place in the absence of oxygen since it inhibits the formation of polymers. The great advantage of this method is that the size of pores may be adjusted much better and separation is much better and this may be achieved by the increase or decrease of the concentration of monomer solution.

Polymerization is made between two glass plates of a size similar to the size of gel. They introduce spacers between them with thickness between 0.75 mm – 1.5 mm depending on the desired thickness of gel. To prevent the pouring out of the gel from between the plates and to obtain a uniform thickness, the plates must be tightly attached to each other by means of some clips before pouring the gel.

The monomer stock solutions are prepared from powder of acrylamide – bisacrylamide in proportion of 29:1. The gel concentrations used generally range between 4 and 20% depending on the type of proteins subject to separation. These concentrations may be obtained by using some different proportions of monomers in the final polymerization solution.

Over the polymerization solution they pour electrophoresis buffer, distilled water and two polymerization agents: ammonium persulphate and TEMED. The solution prepared this way is injected between the two glass plates taking care not to form air bubbles that might negatively influence polymerization and migration. After pouring the gel, in the upper part of the glass plates

they introduce a comb having a similar thickness to that of spacers, comb that may have a variable number of teeth. To form buckets with the completely polymerized walls, the cob is wiped with a piece of cotton soaked in TEMED.

After polymerization usually lasting for to hours at room temperature, the clips are taken out and the comb and spacers are easily removed. The glass plates together with the polymerized gel between them are introduced in the electrophoresis vertical device.

The electrophoresis device is equipped with two tanks having two platinum electrodes. After the introduction of the gel in the device and its airtight fixation, in the tanks they may introduce the buffer solution that may be: Tris HCl, Tris Borate or Tris Acetate (pH =8.6). After the application of samples in the formed gels, the device is closed and connected to a power source since migration occurs at voltages between 40 and 300 volts.

After migration, the gel is taken out from between the two plates and is introduced in a 10% concentration trichloroacetic acid solution. Though the electric field was interrupted, proteins have the tendency to migrate out of inertia and that is why this solution precipitates them and fixes them at the very spot where they are.

Depending on the acrylamide and bisacrylamide concentration encountered for the same gel, gels fall into three types:

1. One concentration gels

They are generally used when the sample to be separated contains molecules having relatively similar sizes, their preparation being made as described above.

2. Discontinuous gels

These gels are very often used since their resolution is much better than that of the previous ones. The work technique is similar but needs several more steps.

The difference consists in the fact that the discontinuous gel is made of two areas: **running gel**, which is prepared similarly to the previous one having a concentration depending on the size of molecules that are to be separated. This is poured between plates until about 80% of the space is full. Then, in the part where it comes into contract with air it is covered with water solution saturated in isopropanol. This helps creating a smooth surface important in the migration process since proteins will start from the same starting point as the separate strips are straight. We must mention that the pH of the buffer used for the preparation of this gel is identical to that of the buffer used for the loading of device's tanks (pH = 8.6).

Meanwhile they prepare the **stacking gel** having a concentration of 4%, the pH of the buffer solution used for the preparation of the stacking gel solution being 6.8.

After the complete polymerization of the separation gel lasting for about 2 hours, the isopropanol solution is removed from its surface by washing with distilled water. In the remained space, they pour stacking gel, they introduce the comb and wait for half an hour for its polymerization.

After applying the samples in the buckets formed, the device is closed and connected to a power source since migration occurs at voltages between 40 and 300 volts.

The pH difference between the two gel portions leads to a discontinuity of the voltage applied causing the migration of molecules together with the separation gel. When they reach the separation gel, they are concentrated and migration in it occurs depending on size, shape and electric charge. The effect of this discontinuous system is the obtaining of some very well individualized strips.

3. Gradient gels

They are successfully used to separate small size proteins, DNA fragments and peptides and to separate the protein mixtures, namely DNA having different molecular sizes.

The acrylamide concentration in these gels is 4% in the upper part and increases linearly towards the lower part reaching 20-25%. The strips obtained in this case are very well individualized due to the decrease of pore size as we get closer to the lower part of the gel, a thing limiting the molecule diffusion.

Depending on the migration conditions, electrophoresis in polyacrylamide gel may take place in a **native** or **denaturant** system.

1. Electrophoresis in native conditions

The protocol presented below may be used for the migration of proteins in native conditions. In this case, they migrate in the electrophoretic field depending on their electric charge, size and shape. The great advantage of this type of migration is that proteins keep their natural structure, the enzymatic activity (in case of enzymes), a very important thing when one ants to make different studies related to their conformation, associations with other proteins and biomolecules, *(fig. 8)*.

2. Electrophoresis in denaturant conditions

It consists in using the denaturant agents in gel and sample: (Urea-PAGE) and β-mercaptoethanol. β-mercaptoethanol is a reducing agent capable to break the disulphidic crosslinks

(in case of proteins) and to reduce polymers to monomers, the urea maintaining molecules in a denaturated state. In this case, proteins lose their native properties.

This type of electrophoresis may be achieved by SDS adding, a detergent that links to proteins and neutralizes their electric charge. In this case, proteins migrate in the electrophoretic field depending on their molecular mass.

In case of DNA migration in denaturant conditions, besides urea they also use formaldehyde as a denaturant agent.

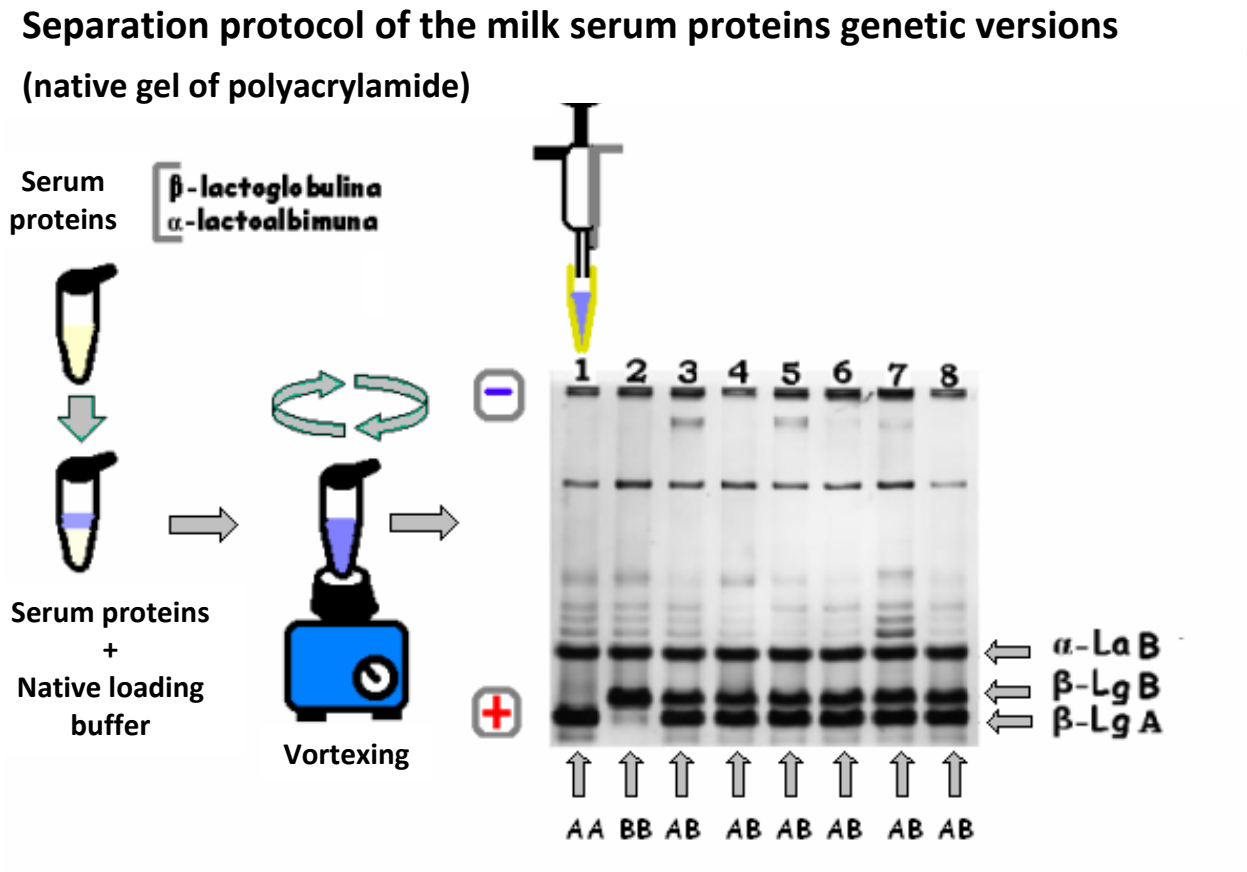

Fig. 8. Electrophoresis of whey proteins from members of the Bovidae family in polyacrylamide native gel (continuous system, gel of one concentration 14.2%). This method may be used to identify alleles A and B of β-lactoglobulin (gel photo Lum, Medrano et al. 1997).

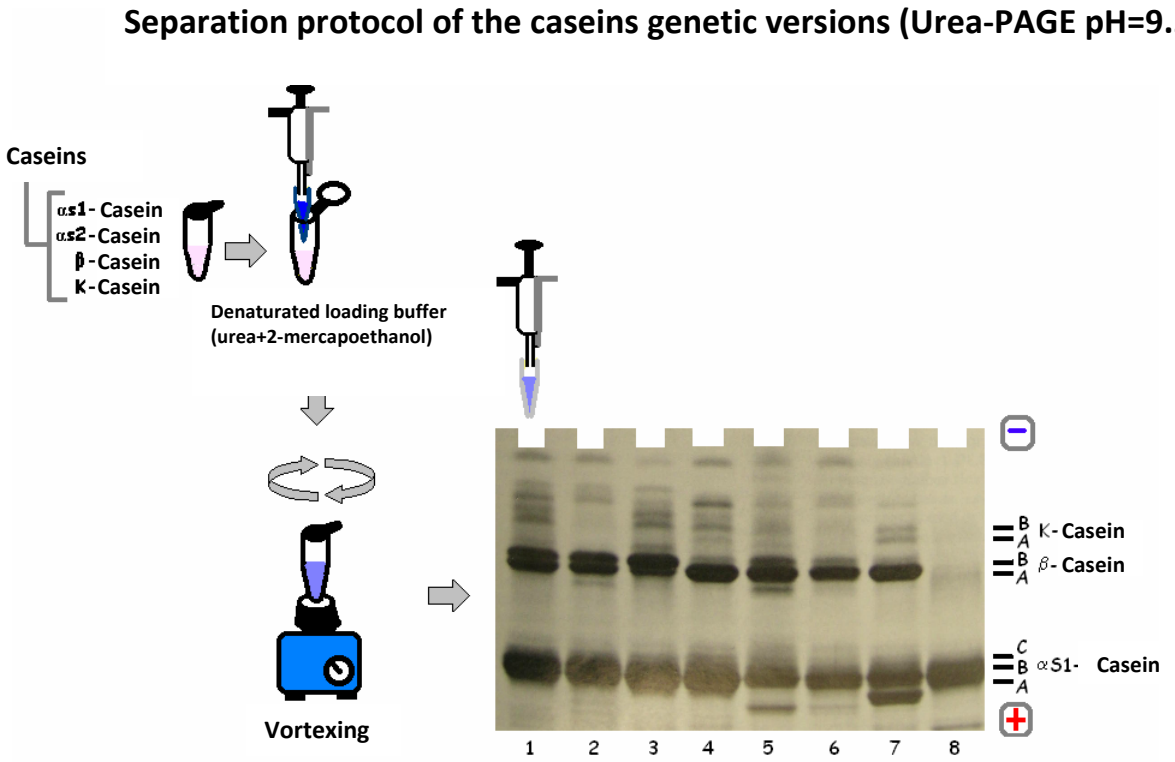

Fig. 9. Electrophoresis of casein fraction from members of the Bovidae family in polyacrylamide denaturant gel, pH=9.5 (discontinuous system: 4.6% stacking gel and 8% migration gel). This method may be used to identify alleles A and B of *K-casein, β-casein as well as of alleles A, B and C of αS1-casein, (gel photo Medrano et al. 1989). 1. K-casein BB; β- casein AB; αS1-casein BC; 2. β-casein AB; αS1-casein BC; 3. K-casein BB; β-casein BB; αS1 casein BC; 4. K-casein AB; β-casein AA; αS1-casein BB; 5. K-casein AB; β- casein AA; αS1-casein BB; 6. β-casein AA; αS1-casein BB; 7. K-casein AB; β- casein AA; αS1-casein AB; 8. αS1-casein BC; Sigma standard.*

Isoelectric focalization method

IEF is an electrophoretic method separating proteins depending on the isoelectric point (pI). Proteins are amphoteric molecules; they may have a positive, negative or zero electric charge depending on the pH of the medium where they are (fig. 10).

The technique was used for the first time in 1984 by Seibert in the study of cow milk protein polymorphisms.

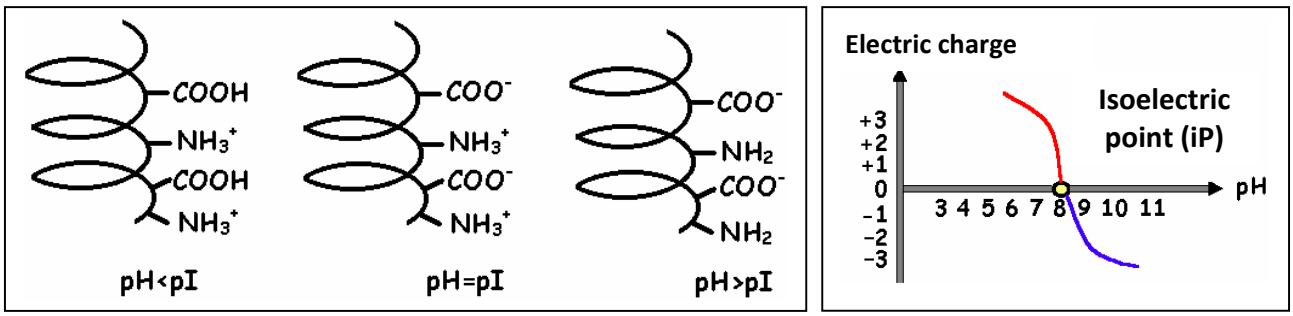

Fig. 10. Schematic representation of the isoelectric point

The electric charge of a protein is given by the sum of positive and negative electric charges characterizing the component amino acids. They present amino groups and terminal carboxyl groups. Depending on the composition in amino acids of the respective protein, the total charge of the protein may be negative or positive.

The isoelectric point is specific to every protean species and the electric charge of the protein is zero in this point. Proteins are positively charged at pH values below their isoelectric point and negatively charged at pH values above their isoelectric point. If the net charge of protein is graphically represented depending on the pH of the medium where the protein is (fig. 10), the resulting curve will intersect the abscissa in the isoelectric point.

The presence of a pH gradient is very important for this technique. In a pH gradient, under the influence of an electric field, a protein will move into gradient towards the spot where its electric charge will be zero. A protein with a total positive charge will migrate towards the cathode (-) becoming less and less positive as it gets closer to the isoelectric point. A protein with a negative charge will migrate towards the anode (+) becoming less negative as it gets closer to the isoelectric point. If a protein could diffuse from its isoelectric point, it might take back its electric charge and migrate backwards. This is the focalization effect used by this technique that concentrates proteins in their isoelectric points and allows their separation based on some small differences of electric charge.

The resolution of this technique depends on the pH gradient slope and the force of the electric field. This technique usually uses very high voltages (higher than 1,000 volts). When proteins reached the isoelectric point in the pH gradient, there is a much reduced movement of ions in the system leading to a very small final current (below 1mA). Isoelectric focalization for a certain sample generally occurs for a steady number of volts-hours.

Migration takes place in horizontal electrophoresis devices. They use polyacrylamide gels of different sizes as a support for the migration of the protein sample. Gels may be prepared from monomer stock solutions or they may be bought readily prepared (with the need to rehydrate them before use) thus eliminating the risk of contamination with acrylamide and bisacrylamide (which are neurotoxic and carcinogen) and a high level of reproducibility is ensured this way.

After the application of samples, the voltage is gradually increased up to the value necessary for focalization and is kept for several hours. An initially low voltage minimizes aggregation of proteins into the sample. The gradual increase of voltage is recommended for large quantities of charged sample.

Visualization of proteins after migration is made by colouring with Coomassie Brilliant Blue or Silver Staining.

Electrophoresis in agarose gel

It is mainly used to separate DNA fragments that were subjected to an amplification process. It may also be used for protein separation.

This method uses agarose as a separation support which is extracted from the red algae of *Rhodophyta* species. The movement of DNA fragments is made under the influence of the electric field and migration occurs in a horizontal system. The DNA is negatively charged and, as a consequence, the samples subjected to migration will be applied in gel in the part towards the cathode. They will migrate through the agarose gel towards the anode depending on their size. Agarose gels have a weaker resolution than the polyacrylamide ones and they may separate fragments between 100 pb – 60 kb depending on the agarose concentration (1- 4%).

The buffer solutions used for the migration of biforked DNA samples contain EDTA with a pH= 8 and Tris acetate (TAE), Tris borate (TBE) or Tris phosphate (TPE). These buffer solutions are prepared as concentrated solutions (10X) and are kept at room temperature. TAE has a smaller buffering capacity than the other two.

The preparation of agarose gels starts from choosing the desired concentration depending on the size of the fragments that are subjected to migration. For example, for a gel concentration of 3%, we weigh 3 g agarose, to which they add 100 ml of TBE 1X. This solution is boiled on steam bath up to the complete melting of agarose and the formation of a homogenous and limpid solution. After a preliminary cooling (not complete), it is poured in the electrophoresis tank and they introduce the comb with a variable number of teeth depending on the gel size. They wait for 30

minutes for polymerization, afterwards they remove the comb and the gel is introduced into the electrophoresis device. They pour the migration buffer TBE 1X over it.

The DNA samples that were mixed beforehand with the loading buffer (containing 500 µl TBE 10X, 400 µl glycerol and 100 µl Bromfenol Blue 2%) in a ratio of 5:1, are then loaded in buckets. The adding of bromfenol blue in the loading buffer helps following the migration front. After closing the electrophoresis device, they apply an electric current of 70 - 75 volts (steady voltage), the migration time being of about 3 hours for a gel concentration of 3%.

The colouring of the DNA fragments may be made by means of the ethidium bromide. This may be included in the agarose gel, before its pouring into the tank. It will migrate opposing DNA, namely towards the cathode and will mingle with its molecules. If migration lasts for a long time, the ethidium bromide shall come out of the gel and the strips weak in DNA will be less visible. We must mention that the ethidium bromide introduced in the gel decreases by about 15% the mobility of DNA fragments. Colouring may also be made after migration since it has the advantage that the electrophoresis devices do not contaminate. The major disadvantage is that the DNA fragments may not be visualized during their migration. The examination of the gel is made in UV light, with a length of 302 nm. Taking photos of the gel for subsequent exams is made in Polaroid system by means of a digital camera.

Techniques used to identify milk protein polymorphisms at DNA level

PCR (Polymerase Chain Reaction) Technique

It relies on polymorphism at allelic level in terms of length of the amplification product obtained by means of 2 specific primers complementary of ends 3' of the DNA sequence that is to be amplified. The amplification products are separated by electrophoresis in agarose or polyacrylamide gel and after coloring with ethidium bromide they are visualized in ultraviolet light when a fluorescent coloration appears in the area where migrated DNA fragments exist.

Polymorphism may be very high when primers are complementary to some unique DNA sequences flanking the respective DNA. When polymorphism is low, the PCR technique may be completed with *RFLP (Restriction Fragments Length Polymorphism)* also called *CAPS (Cleaved Amplified Polymorphic Sequences)* supposing the enzymatic digestion of the amplification products.

The amplification and digestion products are visualized in the polyacrylamide gel by argentic colouring.

PCR markers are codominant since there is the possibility to differentiate homozygotes from heterozygotes.

Work technique

Amplification mixture *(PCR master mix),* must contain the following components:

1. PCR 10 X Buffer – is a buffer solution maintaining pH constant during the amplification reaction and it is specific to every polymerase;

2. $MgCl_2$ 25mM solution – has the role of mediating the fixation process of primers and the enzymatic activity;

3. Deoxyribonucleotide triphosphates 10 mM (dATP, dCTP, dGTP, dTTP) are used by polymerase in the elongation process;

4. DNA polymerase 5 U/µl – it is a thermostable enzyme at 95^0 C being the one that makes elongation. Depending on the bacteria from which it was extracted, we may have:

-Taq polymerase produced by the thermophilic bacteria *Thermus aquaticus*

-Tth polymerase produced by the thermophilic bacteria *Thermus termofilus;*

5. Specific primers are usually used in concentration of 10pmoles/µl;

6. The mixture is completed with **bidistilled water** up to 24 µl (for a reaction of 25µl);

7. DNA mould whose optimal concentration may range between 30 - 300ng/µl. Optimal purity ranges between 1.7-1.8. Inferior purities do not significantly affect amplification.

The samples prepared this way are introduced in thermocycler, a device that may be programmed to automatically change temperature at different stages depending on the desired thermal profile.

Amplification occurs in three stages, the second stage being made in its turn of three sub-stages repeating 35 times. Following these amplification stages, the target fragment is amplified in millions of copies.

1. DNA predenaturation consists in heating the reaction mixture at 95^0 C and maintaining this temperature for at least 3 minutes. In this stage occurs the breaking of hydrogen bridges between the complementary catenae resulting monocatenar DNA;

2. Denaturation occurs at 94^0 C for one minute and aims at completely separating the biforked DNA chains;

3. Primers' fixation usually takes place at 45 – 60^0 C, depending on its composition in nitrogenous bases and its size. These specific primers may have sizes of 20 – 30 pb being complementary to ends 3' of the target DNA sequence;

4. Extension (Elongation) occurs at 72^0 C for 1 - 3 minutes depending on the length of the desired amplification product. Taq polymerase functions at an optimal capacity at this temperature. Elongation consists in the extension of the target DNA sequence from the primers' fixation place. The effectiveness of the extension product is 1 Kb/minute;

5. Final extension occurring at 72^0 C for 7 minutes aims at finalizing the extension process;

6. Sample storage taking place at 4^0 C for 24h and for several months at -20^0 C.

Then these samples shall be subjected to migration in agarose or polyacrylamide gel.

PCR–RFLP (Restriction Fragments Length Polymorphism) Technique or CAPS (Cleaved Amplified Polymorphic Sequences)

The two techniques combined were and still are successfully used to identify alleles that suffered modifications at the level of restriction sites by mutation or restructuring at their level: deletion, inversion, translocation of some fragments. RFLP technique relies on polymorphism at allelic level in terms of size and number of restriction fragments obtained after digestion with restriction enzymes of the PCR amplification products.

The restriction enzymes cut DNA in specific sites (restriction sites) where they meet short inversed repetitions of nucleotides called palindromic sequences (table 7).

These enzymes are produced by bacteria defending this way against the bacteriophages' attack. When the DNA phage enters the bacteria, it produces restriction. Restriction does not occur when the restriction site is methylated (adding a methyl group to adenine or cytosine from the structure of the restriction site). Every bacterial strain produces a specific restriction enzyme recognizing a certain palindromic sequence and cuts only in that restriction site (table 7).

The genes codifying the different restriction enzymes were cloned in E.coli which currently produces these enzymes in large quantities.

*The PCR-RFLP method needs to go through the following **work stages**.*

The DNA extracted from the sample under analysis is subjected to a PCR amplification process with specific primers that are complementary to ends 3' of the DNA sequence that is to be

amplified. The amplification product is then subjected to a digestion process with one or several restriction enzymes.

Table 7

Several restriction enzymes, their recognition sites and the bacteria where they were discovered

Enzyme	*Source bacteria*	*Restriction site*
EcoR I	Escherichia coli	▼ 5’ G--A-A-T-T-C 3’ 3’ C-T-T-A-A--G 5’ ▲
EcoR II	Escherichia coli	▼ 5’ G--C-C-T-G-G-C 3’ 3’ C-G-G-A-C-C--G 5’ ▲
Hinf I	Haemophilus influenzae	▼ 5’ G--A-N-T-C 3’ 3’ C-T-N-A--G 5’ ▲
Hind II	Haemophilus influenzae	▼ 5’ G-T-Pi--Pu-A-C-3’ 3’ C-A-Pu--Pi-T-G 5’ ▲
Hind III	Haemophilus influenzae	▼ 5’ A--A-G-C-T-T 3’ 3’ T-T-C-G-A--A 5’ ▲
Hae III	Haemophilus aegypticus	▼ 5’ G-G--C-C 3’ 3’ C-C--G-G 5’ ▲
Mae II	Methanococcus aeolicus	▼ 5’ A--C-G-T 3’ 3’ T-G-C--A 5’ ▲

Choosing the adequate enzyme needs knowledge about the amplified sequence in terms of number and position of the specific restriction sites. This is of maximum importance when one wants to discriminate between two allele genes out of which one suffered mutations at the level of a certain restriction site. This may be known by a sequencing process, an important stage in establishing the adequate restriction enzyme that is to be used in the genotyping process. The use of some inadequate enzymes for the respective DNA fragment may lead to the digestion of alleles in an

identical way resulting fragments of similar sizes (though the same electrophoretic profile) or the non-digestion of these amplified fragments if there are no restriction sites on them for the respective enzyme.

The digestion process generally occurs at 37^0C for a couple of hours or one may also use the overnight digestion, a case where they will obtain the same electrophoretic profile without registering the supra-digestion process.

The resulted fragments are separated in agarose gel and visualization is made by colouring with ethidium bromide in ultraviolet light.

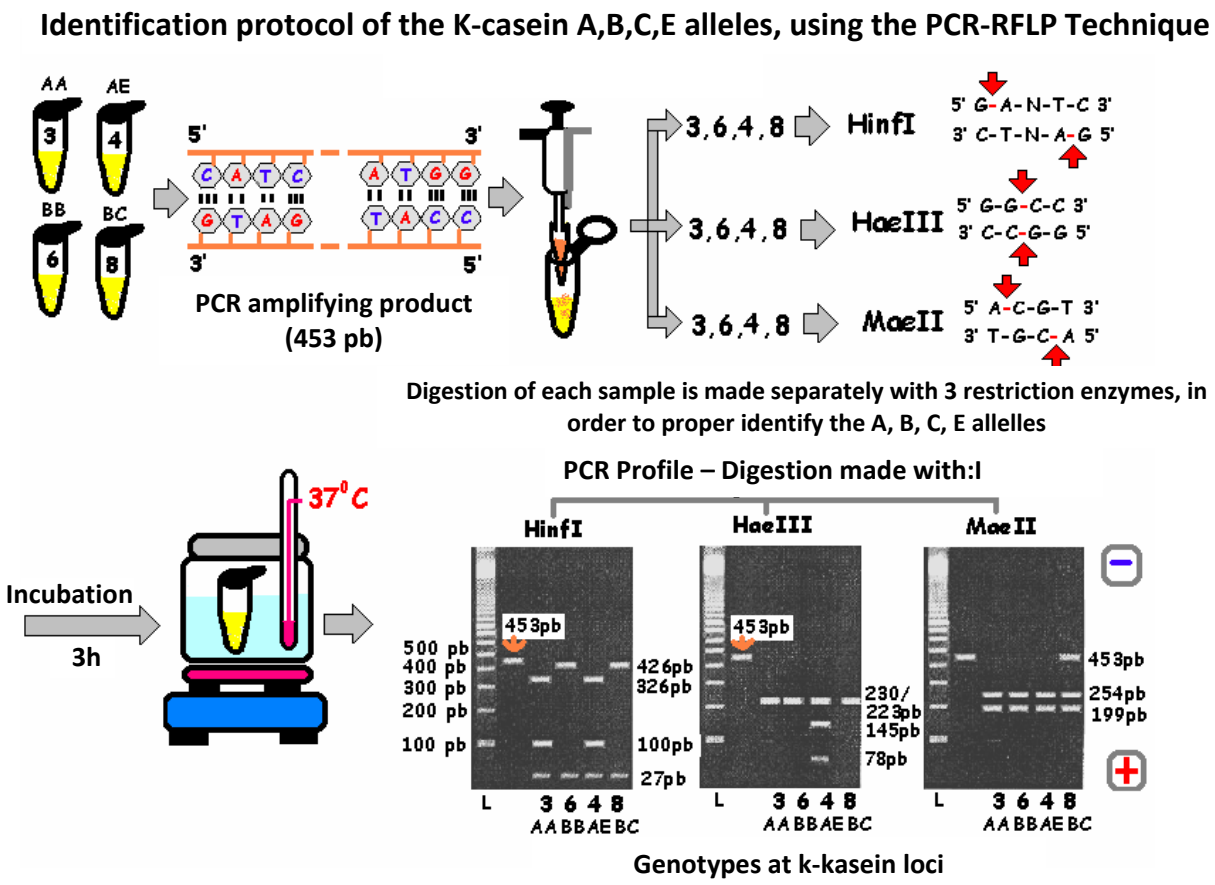

Fig. 11. Principle of PCR-RFLP technique. Identification protocol for alleles A, B, C and E of K-casein by digestion of every sample separately with 3 restriction enzymes

PCR use in animal science

Since its discovery *(Kary Mullis, 1985),* PCR technique and its variants have found a large applicability in the field of *farm animal improvement assisted by genetic markers (MAS),* allowing the determination of genotype for a certain locus before a certain character was expressed phenotipically.

For example, in the past, the determination of genotype for individuals from F1 or F2 presenting a dominant phenotype was made by their retrobreeding with the recessive parent. If the segregation ratio from R1 is 50:50 %, it meant that the individual for which we wanted to determine the genotype is heterozygote for the respective locus. If in R1 we had 100 % individuals with a dominant genotype, it means that the individual for which we wanted to determine the genotype is homozygote for the respective locus. Though this work methodology gave results, the time necessary for the determination of genotype is very large for the farm animals (several years).

PCR technique comes to substantially reduce the interval between generations and the time necessary for the determination of a certain genotype. The work methodology is the one presented above needing just small DNA quantities from the individual for which we want to establish the genotype for a certain locus and some quite accessible equipment. The determination of a genotype for a certain locus usually lasts a couple of hours.

The great advantage of this technique is that it allows the determination of genotype of a certain individual for a certain locus, regardless of age (embryo, fetus, youth, adults), sex, physiological state or environment conditions.

A new work methodology has developed based on this technique in the improvement of farm animals, technique called *MAS (genetic marker assisted selection)* completing the classical improvement diagrams.

In the classical improvement, the correct establishing of the improvement value for an individual involves the knowledge of very many data related to own performances, ascendants, collaterals and descendants of such individual. All these data are centralized, the method for the establishing of this improvement value being knows as BLUP.

Marker assisted selection (MAS) sustains BLUP so as to establish the improvement value as correctly as possible.

The researches effectuated highlighted a series of genetic markers that are correlated with the quantitative and qualitative milk yield. Thus, the alleles of K-casein and alleles of β-lactoglobulin are the main genetic markers taken into account when establishing the improvement

value. We determined that BB genotype for K-casein locus is correlated with a large quantity of casein and milk having superior processing properties. BB genotype for β-lactoglobulin locus is correlated with a larger quantity of casein and fat and AA genotype with a larger milk yield.

The genotypes of these 6 loci codifying the major milk proteins may be determined by the techniques presented in this chapter.

The goals and activities for 2008 year have been fully acomplished

PROJECT MANAGER,

A/P Creangă Ştefil, Ph.D.