Interrelation between nano and micro levels in genome organization

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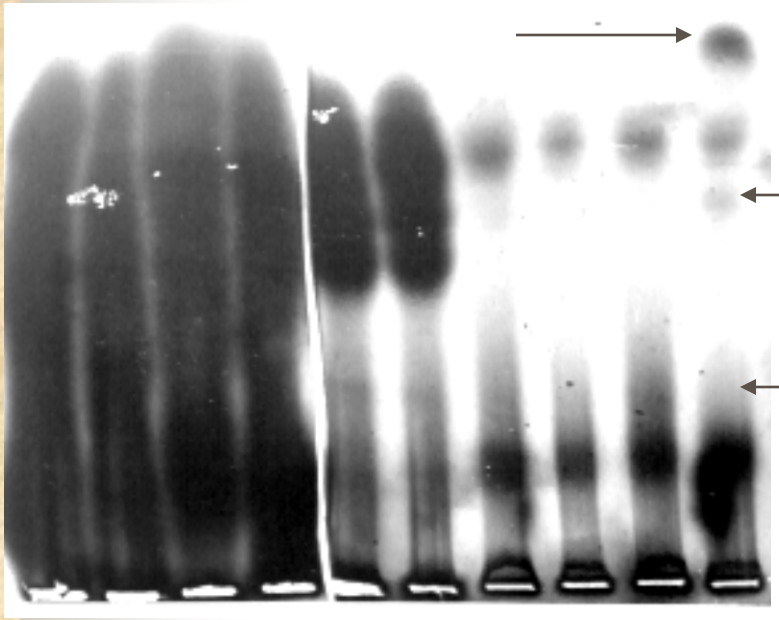
The employment the molecular-genetic methods in investigation, improving and conservation of domestic and close related wild species of animals and plant

- Molecular-genetic markers:
- Structural genes (protein polymorphism, RFLP), DNA biochips
- RAPD-PCR, ISSR-PCR
- Cytogenetic researches

Hopes and results of DNA technology using in species improving

- Transgene herds and "bioreactors"
- Difficulties:
- Problems in obtaining the predicted quality and quantity of transgene products
- Problems in obtaining and using of stem embryonic cellular lines for transformation

INTEGRATION OF THE TRANSGENE CONSTRUCTIONS IN RABBITS LEADS TO CHANGES OF THE GENE EXPRESSION.



1 2 3 4 5 6 7 8 9 10

Tissue-specific esterase spectra: 1,2 – liver, the control; 3,4 – liver, transgene rabbits; 5 – muscles, the control; 6 – muscles, transgene rabbits; 7,8 – cardiac muscle, the control; 9, 10 – cardiac muscle, transgene rabbits

The differences between transgene animals (LacZ under β-casein cattle promoter; cattle somatotropin) and their sibs in the expression of tissue-specific allozyme spectra (ME, ES D and fast moving zone of esterase - ES) were revealed. The most expressive differences observed in lungs and hearts.

The similar changes were observed in animals which obtaining the high level of ionizing irradiation.

It was in accordance of the data of revealing of chromosome ionizing markers in transgenic pig (Klenovitskiy et al.) .

Techniques of embryo transplantation and the problems of natural selection

- 1) The relation between quantity of washed embryos and the cytogenetic instability of peripheral blood cells of cows - embryo donors was determined.

- 2) As the result: the genetic structure of the offspring received after transplantation of embryos differed from typical for parental breed: the part of gene pool was eliminated (the data were obtained on Aberdin-Angus herd, obtained after embryo transplantation and comparisons with parent animals).

Correlation between different traits of chromosome apparatus instability in blood cells of cow – embryo donors and embryo quantity

Correlation between embryo quantity and cytogenetic anomaly frequencies in cell blood of embryo donor cows – r= 0.58; p<0,05

Chromosome instability traits	AI	AII	PP	RB	CA	ACF	embryo quantity
Aneuploidy AI (59-61)	1.00						
Aneuploidy AII (53-63)	-.39	1.00					
Polyploidy PP	-.23	.34	1.00				
Robertsonian translocation RB	-.43	.45	.61	1.00			
Chromosome aberration CA	.37	-.29	-.38	-.58	1.00		
Asinchronous cchromosome fissing ACF	-.07	.09	.01	-.09	-.40	1.00	
embryo quantity	-.15	-.24	-.43	-.31	.58	-.45	1.00

Conclusion N1

Different artificial manipulation with genomes lead to “switch” on the new selection factors, changed all our planes and perspectives

Quantity trait loci (QTL) mapping

- Mapping of QTL of dairy efficiency (the milk yield, % of fat, % of protein) in Holstein cows lead to different results in researches of herds in the different countries even if it was carried out on the same bull lines - the main genes were localized totally in 14 autosomes from 29 available in different investigations.
- The reasons: the key genes for complex trait realization varied in connection of changes in animal's feeding, the maintenance and reproduction of animals on different pathogenic backgrounds, and ecological conditions.

Chromosomes in witch localized the QTL of dairy efficiency in Holstein cows in different investigation						Authors
Yield	% protein	protein in summary	% fat	fat in summary	Somatic cell quantity	
-	-	-	2, 15	2, 15	7	Weller J.I. et al., 1990
21	-	21	-	-	-	Ron M. et al.,1994
1, 6, 9	6, 20	1, 9	6	-	-	Georges M. et al., 1995
-	6	-	-	-	-	Spelman R.J.et al.,1996
6, 9	-	6, 9	-	6, 9	-	Wiener P et al.,2000
6	6	-	6	-	-	Olsen HG et al., 2002
7, 29	-	3, 6	-	3, 14, 29	21	Rodriguez-Zas SL et al.,2002
5, 14	5	26	-	19, 26	2, 19	Bennewitz J et al., 2003
-	-	-	-	-	18	Freyer G, et al., 2003

The new hope for revealing of key genes for important traits was the use of the DNA microarray technologies, which allowed to carry out simultaneously the monitoring of nucleotide sequences of the many genes and to estimate activity of their transcription (profiles of gene expression).

PROFILES OF GENE EXPRESSION

DNA microarrays

- spots: oligonucleotides in 70 nucleotide length

- two-color detection (Cy3 / Cy5)

Method:

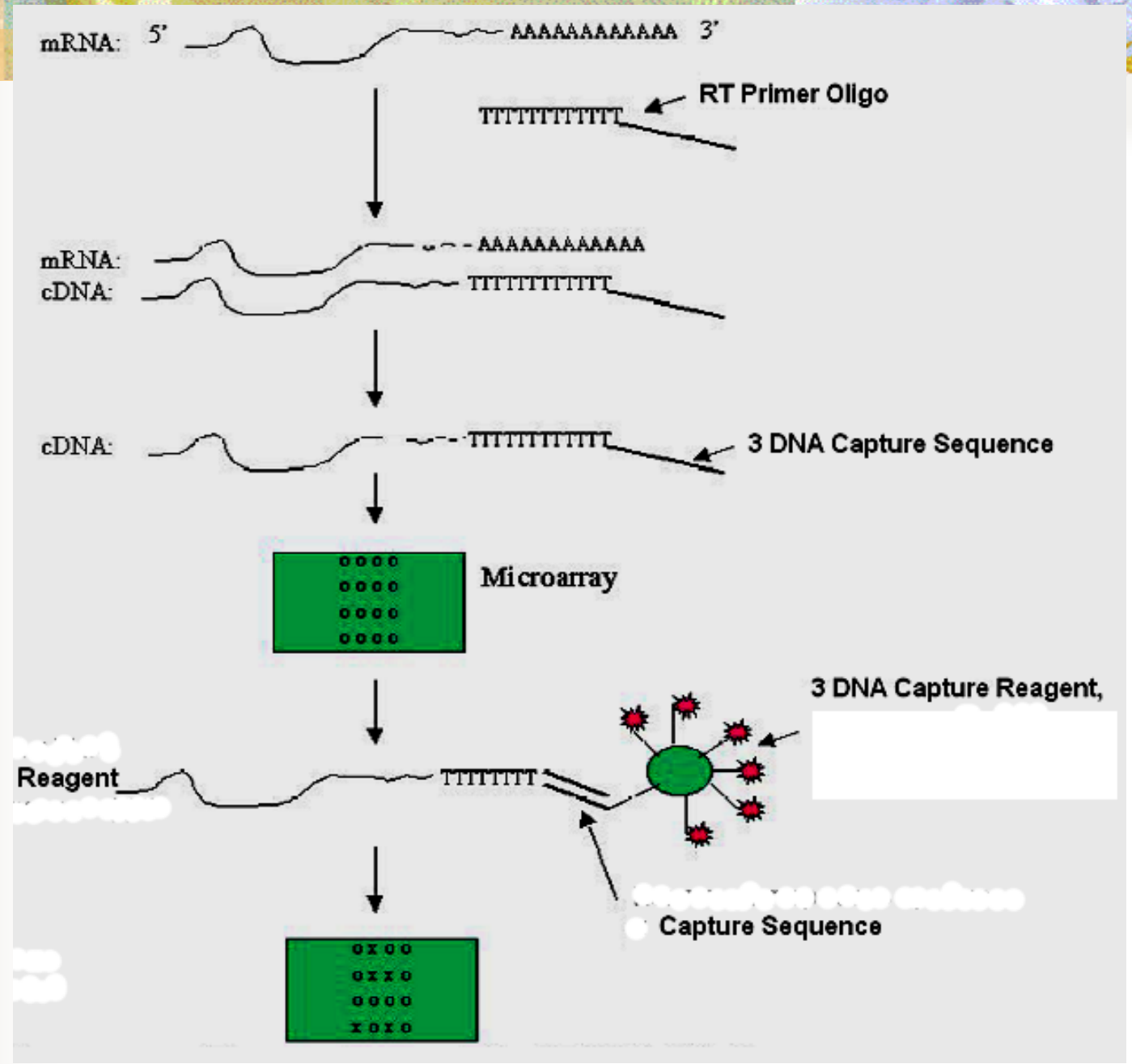
1. Total RNA obtaining

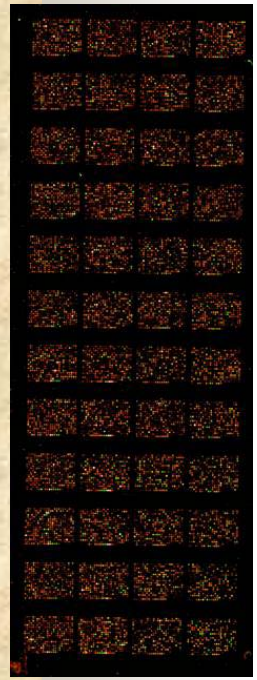
2. cDNA obtaining in RT-PCR with the use of primer of polyT and oligonucleotides for following links to Cy3/Cy5

3. Hybridization with DNA microarray spots

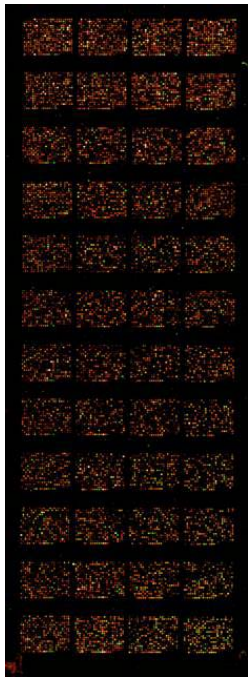
4. Hybridization with 3DNA reagents (Cy3/Cy5)

5. Scanning

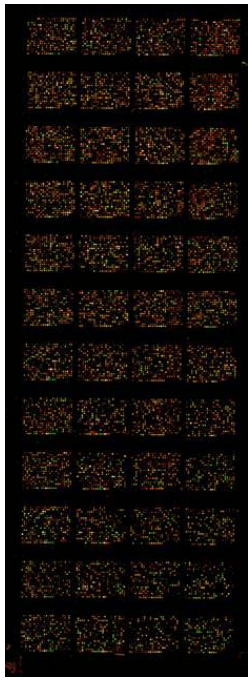

mRNA: 5'
AAAAAAAAAAAA 3'
RT Primer Oligo
TTTTTTTTTTTT
mRNA:
cDNA:
AAAAAAAAAAAA
TTTTTTTTTTTT
cDNA:
TTTTTTTTTTTT
3 DNA Capture Sequence
Microarray
3 DNA Capture Reagent,
Reagent
TTTTTTTT
Capture Sequence



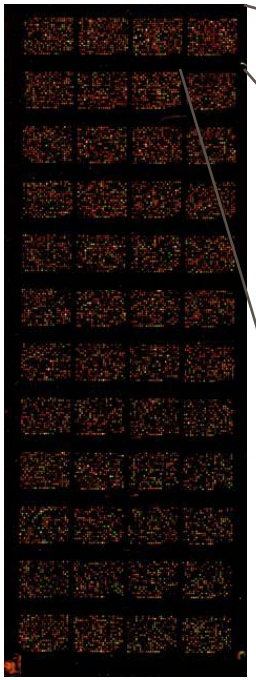
Liver 12016
Kidney 12016



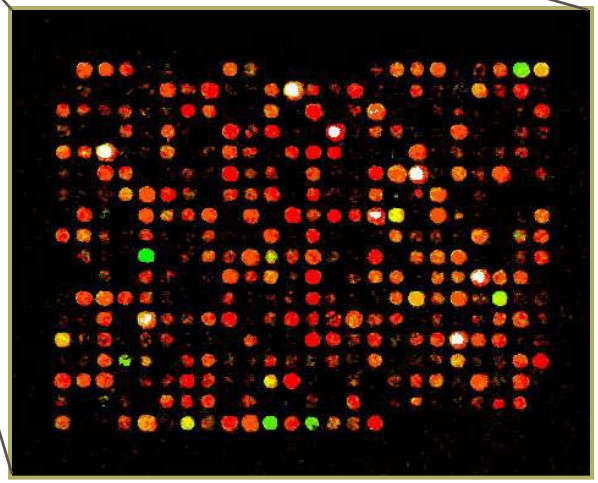
Liver 11940
Kidney 11940



Liver 12139
Kidney 12037



Liver 12294
Kidney 12037



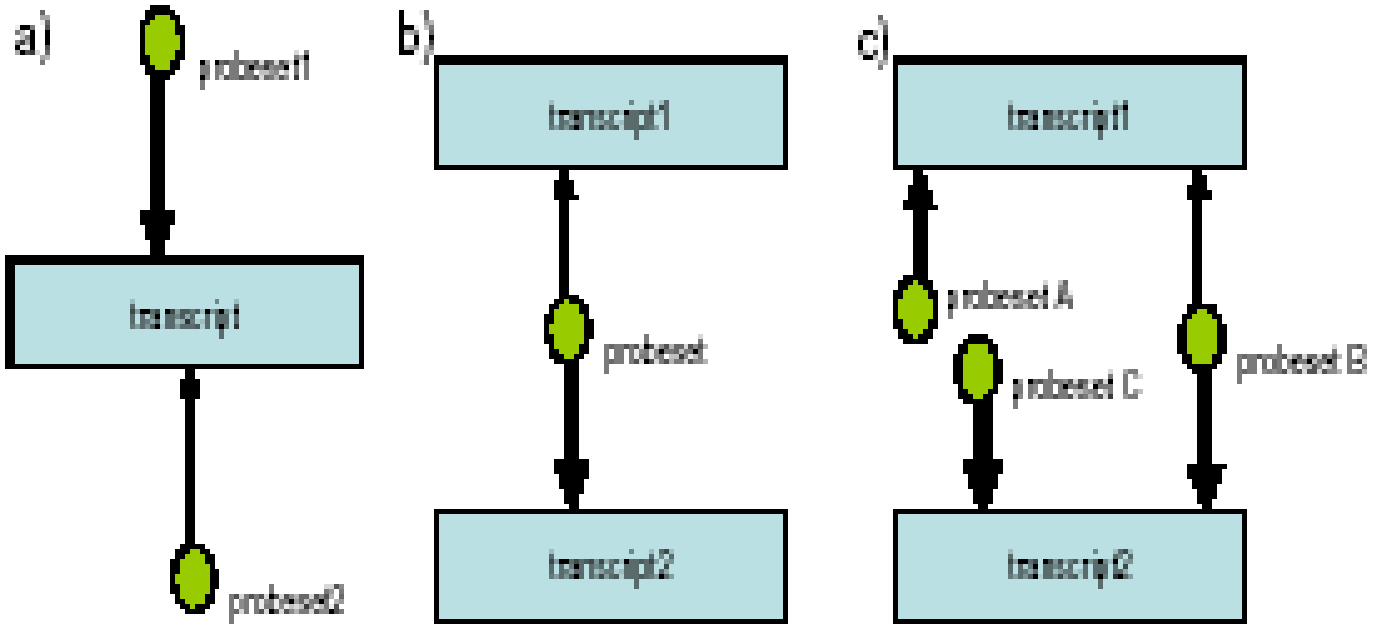
- At the total analysis of 600 genes of the liver, with the maximum distinctions in intensity of hybridization between liver cDNA and kidney cDNA, 12 genes were found out with more than one probe in microarray.
- The gene group with greatest distinctions (more than 10000 standard units of a luminescence) between signal strength of hybridization of various sites cDNA of same mRNA included two genes – Alpha-1-antichymotrypsin precursor (ACT) and Fibrinogen alpha chain precursor. Observable differences were reproduced in independent experiments for all investigated animals.

- Among profiles of gene expression in kidneys for 600 genes, with the maximum distinction in intensity of hybridization between kidney cDNA and liver cDNA, 9 genes were allocated with more than one probe to different sites of the same gene on the microarray.
- The group of genes, which internal sites of hybridization differed more than on 10000 standard units of a luminescence, consisted of the genes coding ATP synthase a chain, Chromogranin A precursor (CgA), Pituitary secretory protein I (SP-I) and Ubiquitin.

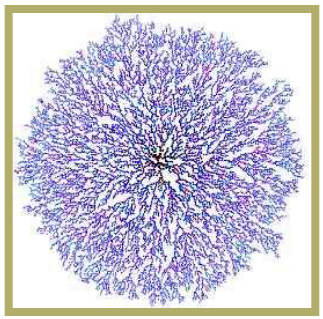
- The search a homology sites to microarray probes was executed with the use of BLASTn in a *Sus scrofa* EST databank presented in NCBI.
- **All considered cases of reproduced differences between hybridization intensity of different microarray probes to cDNA the same gene were typical for the genes belonging to supergene families.**
- **For all cases the expressed differences between homologous sites to different probes for the same cDNA were observed on the quantity of homology fragments, on the presence of homologous sites for other genes, including paraloges.**

The basic motifs of multiple targeting: a) 2 probes 1 transcript; b) 1 probe 2 transcripts; c) a simple combination of a and b variants.

In below - the motifs form the basic building blocks of multiple targeting networks. The strength of relationship between a transcript and a probeset is dependent on the number of probes matching to the transcript.



Okoniewski M.J., Miller C.J. Hybridization interactions between probesets in short oligo microarrays lead to spurious correlations// BMC Bioinformatics 2006, 7:276-289



In spite of multiple sources of mistakes (cross-hybridization, substrate and endocrine regulation of gene expression and so on), in general, the comparison of organ-specific profiles of gene expression in liver and kidneys of pigs had shown, that differences between them in expression of 40 genes corresponded to known organ-specific physiological functions (kidney – control of ion balance in blood; liver – transport proteins), and also histology (in particular, in kidneys the dynactin expression, which participating in the cytokinesis control, was on some orders above in comparison with polyploid liver).

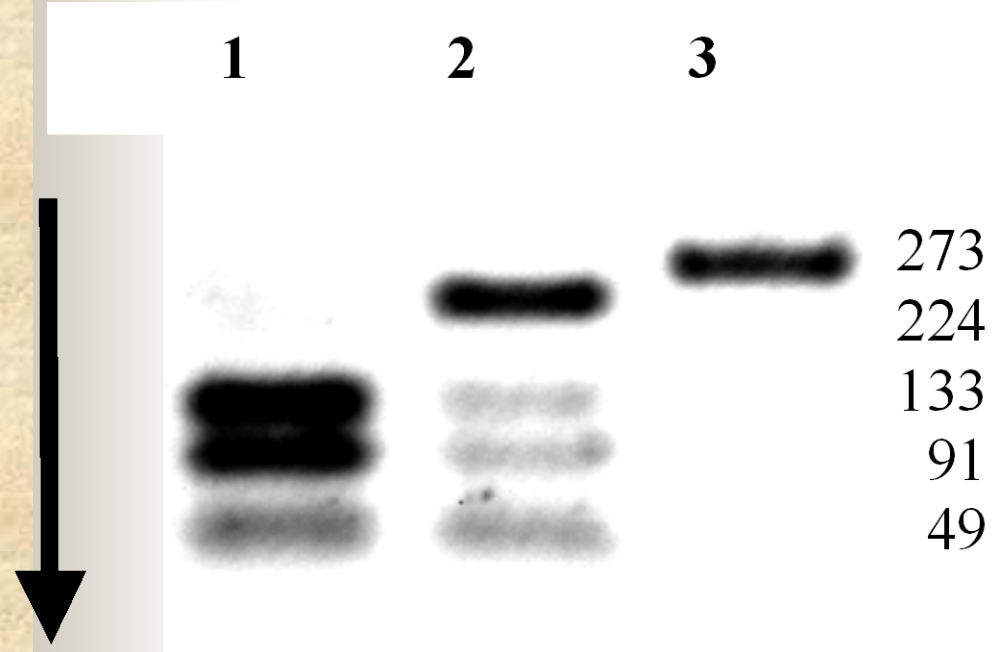
Conclusion N2

The randomness search the genetic targets, related with the productivity trait realization with the use methods of QTL mapping, analyzing of gene expression profiles lead to accumulation many varied data, which could be analyzed and used with hard and in limited cases.

- **Another hope for direct using of DNA methods in selection of domesticated species – it is the find out the gene – candidates of control of productivity traits, related with the physiological systems, directly participated in their realization (for example for dairy efficiency – genes, controlled the milk protein synthesis, hormonal regulation of growing and lipid metabolism).**

Polymorphism of structural genes directly related with the milk production

Polymorphism of the genes, coding of milk proteins



1– homozygous animal with the BB genotype (fragment length 133, 91 and 49 b.p.);

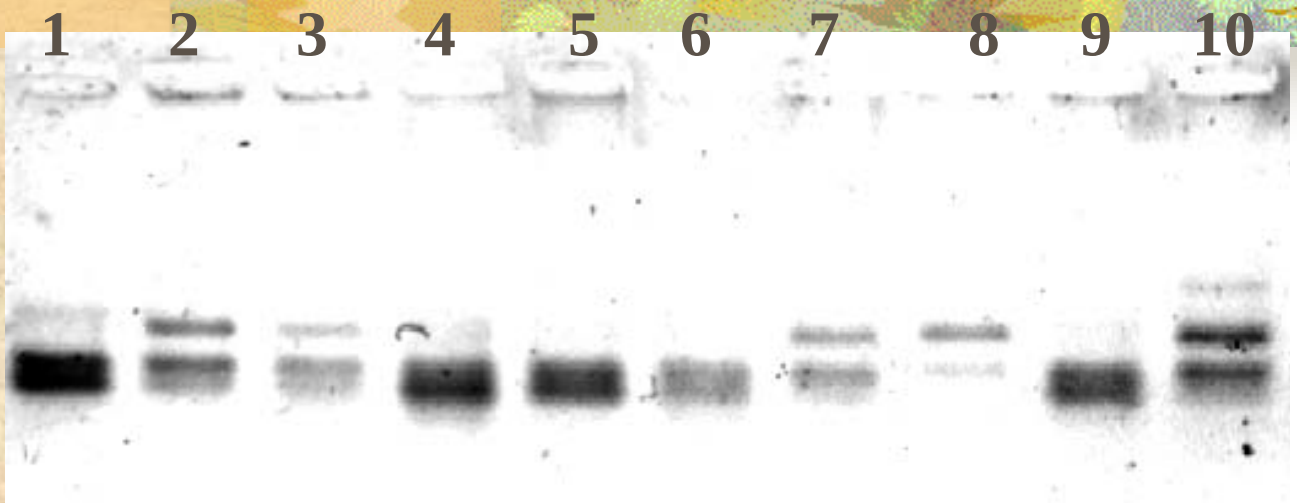
2-heterozygous animal with the AB genotype (fragment length 224, 133, 91 and 49 b.p.);

3 – unrestricted PCR product (273 b.p.).

Kappa-casein gene fragment polymorphism after restriction by restrictase *Hinf I.*

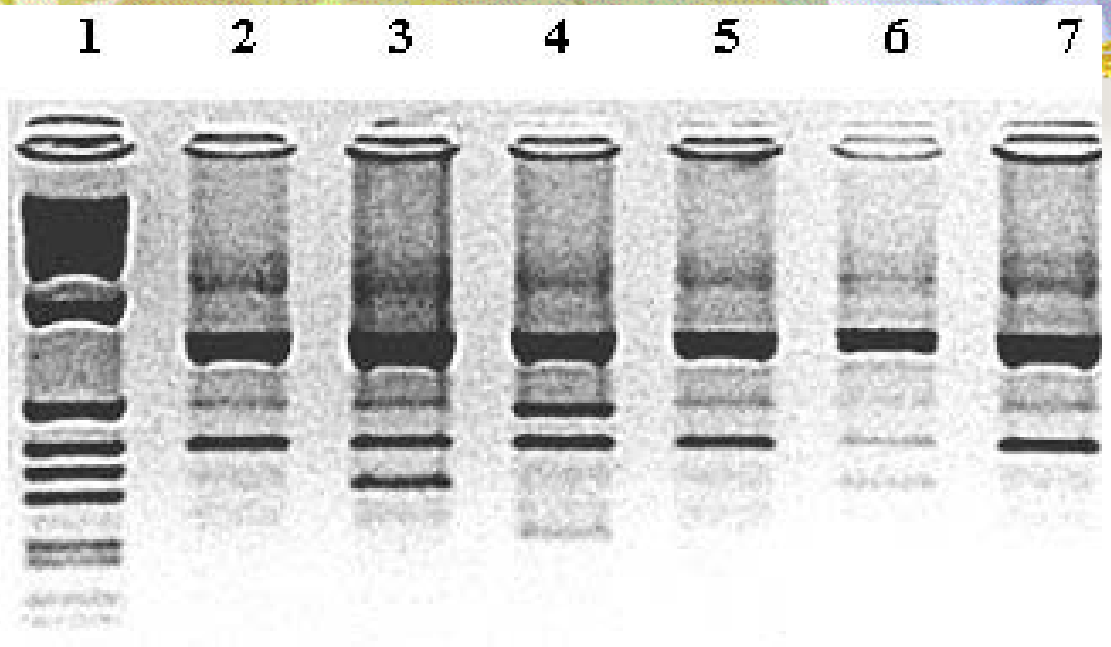
Distribution of kappa-casein alleles A and B in different cattle breeds

Породные группы	Genotype frequencies (%)			Allele frequencies		The animal numbers
	AA	AB	BB	A	B	
Holstein herd 1	64	30	6	0,792	0,208	40
Holstein herd 2	79	21	-	0,895	0,105	46
Holstein herd 3	48	45	7	0,707	0,293	33
Holstein Chernobyl	42	39	19	0,613	0,387	31
Grey Ukrainian	14	72	14	0,500	0,500	22
Red Gorbatovskaya 1	31	44	25	0,531	0,469	22
Red Gorbatovskaya 2	20	47	33	0,433	0,567	16
Red Gorbatovskaya 3	18	62	20	0,492	0,508	61
Yaroslavskaya	15	53	32	0,421	0,579	52
Kostromskaya	35	50	15	0,596	0,404	52
Red Steppe	33	42	7	0,628	0,371	70
Black Wells	24	72	4	0,603	0,397	40

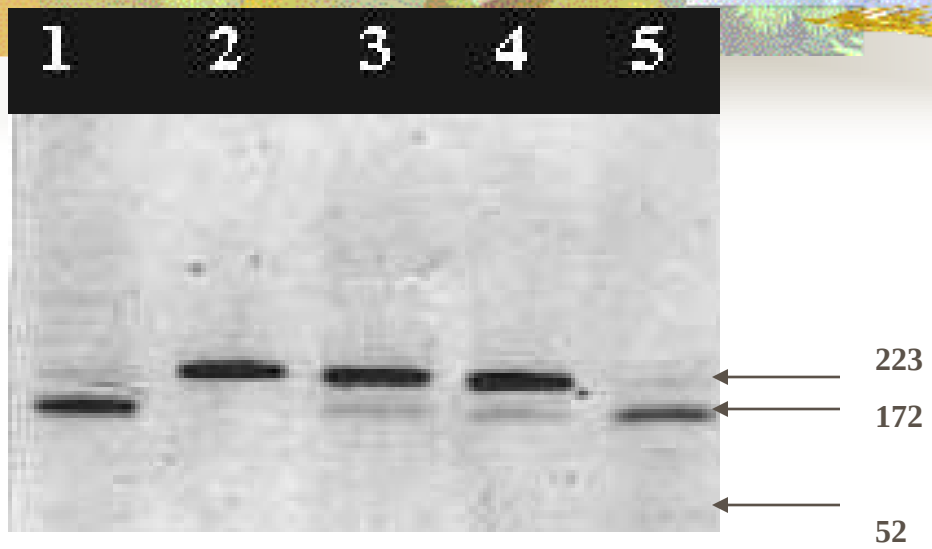


Analysis of restriction of PCR products of β-lactoglobulin gene fragment. Lines 1, 4, 5, 6, 9 – homozygous animals with the genotype BB (fragment lengths in 99 and 2x74 b.p.); 8 – homozygous animal with genotype AA (fragment lengths 148 and 99 b.p.); 2, 3, 7, 10 – animals with genotype AB (fragment lengths 148, 99 and 2x74 b.p.).

The carriers of genotype AA had on 28% more milk serum proteins, on 7% low caseins, on 11% more fat, on 6% low dry mass and more yield in comparisons to animals with BB genotype.

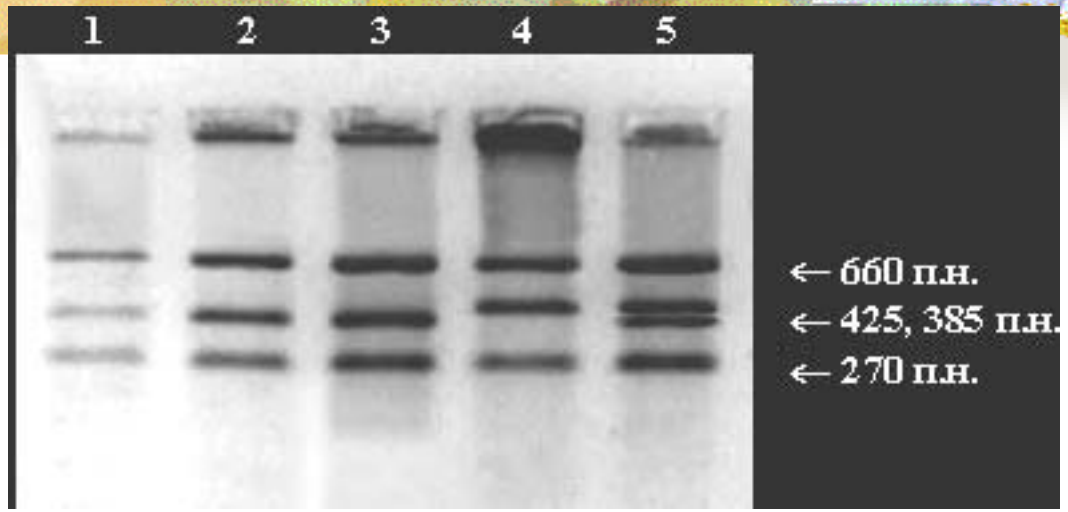


Analysis of restriction of PCR products of cattle leptin gene (hormone of lipid metabolism). 1 – marker of molecular mass; 2, 5, 7 – homozygous animals with genotype AA (fragment lengths 740, 690 and 400 b.p.); 3, 6 – heterozygous animals with genotype AB (fragment lengths 740, 690, 400, 310 and 90 b.p.); 4 – animal with rare genotype AC (740, 690, 470, 400 and 220 b.p.).



Analysis of restriction of PCR products of somatotropin (SH) gene fragments.

Lines 1, 5 – homozygous animals with genotype LL (fragment lengths 171 and 52 b.p.); line 2 – homozygous animal with genotype VV (fragment length 223 b.p.); lines 3, 4 – heterozygous animals with genotype LV (fragment lengths 223, 171 and 52 b.p.).



Analysis of restriction of PCR products of cattle Pit-I gene fragment (factor of transcription regulation of SH gene).

Homozygous animals with genotype BB (fragment lengths 660, 385 and 270 b.p.) – lines 1-3, with genotype AA (fragment lengths 660, 425 and 270 b.p.) – line 4; heterozygous animal with genotype AB (fragment lengths 660, 425, 385 and 270 b.p.) – line 5.

Result: distribution of allelic variants on 5 loci (kappa-casein, beta-lactoglobulin, leptin, somatotropin, the factor of transcription regulation of somatotropin PIT1 between breeds corresponded to the dairy breed differenced from dual-purposes and beef breed on the higher allele frequencies which associated with milk yield, as it was revealed also in independent researches ("dairy" alleles).

However, it was not revealed statistically authentic ASSOCIATIONS between "dairy" alleles on the different investigated loci in INDIVIDUAL COWS.

Gene modules with the same target of quantity trait, for example, milk yield

Module of micelle casein genes – Ca transport (A allele of kappa-casein gene)

Unlinked polymorphism of the genes, coding of milk proteins

Module of milk serum proteins (A allele of β-lactoglobulin gene)

Module of regulation of animal growth (somatotropin - SH; PIT1 - factor of transcription regulation of SH gene)

Module of lipid metabolism - hormone leptin

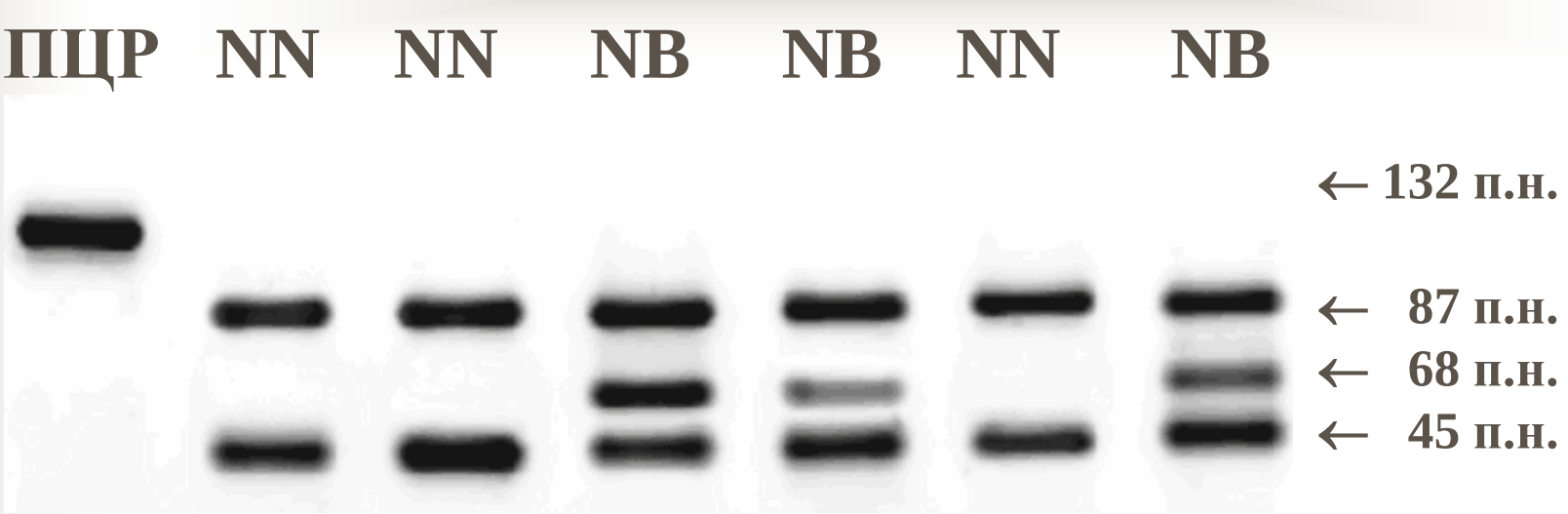
Unlinked polymorphism of the genes, coding of protein hormones, participated in regulation of animal growth, protein and lipid synthesis

Conclusion N3

For desirable expression the same trait (the general yield of milk, for example) the critical alleles could be present in different loci (genes of milk proteins or endocrine regulation) even in the same line of animals.

- **The more effective DNA methods, which can be directly use in practical work, is the revealing of genetically determined diseases, the forecast of their occurrence, working out the methods to management of their distribution**

- **The forecast: the increase number of animals in breed related with the increase probability of the unfavorable mutation occurrence and the high speed of their distribution in breed**
- **Example: Holstein breed, distribution of mutation BLAD (Bovine Leukocyte Adhesion Deficiency)**



Electrophoretical analysis of PCR products of CD 18 gene fragment after restriction by restrictase Hae III.

Genotype of health animals (normal - NN) presented by restricted fragments CD18 gene lengths in 87 and 45 b.p.; heterozygous animals on the carrying of the BLAD mutation (NB) – fragment lengths in 87, 68, 45 and 19 b.p.

The quantity of BLAD mutation carriers in Russian and Ukrainian Holstein

Economy	Quantity of animals	BLAD carriers	BLAD carriers in %
“Progress” (Zolotonosha, Ukraine) 1996 y.	30	1	3,3
Breeding Center (Pereyaslov-Khmelnick, Ukraine) 1996 y.	80	2	2,5
Eleveier, Dnepropetrovsk region, Ukraine, 1995 y.	80	3	3,8
Moscow region, Russia, 1998 y.	36	0	0
Askania-Nova, Ukraine, 1998 y.	90	13	14,3
Breeding Center (Pereyaslov-Khmelnick, Ukraine) 1998 y.	28	1	3,6
Breeding Center, Borispol, Kiev region, Ukraine, 1999 y.	25	0	0
Poltava region, Ukraine, 2003 y.	28	0	0
Knyazhichi, Kiev region, Ukraine, 2003уг.	30	5	16,7

- **During time of the researches the frequency of meeting of BLAD mutation carriers in economies of Ukraine had consistently increased from units of percent on herd to several tens.**

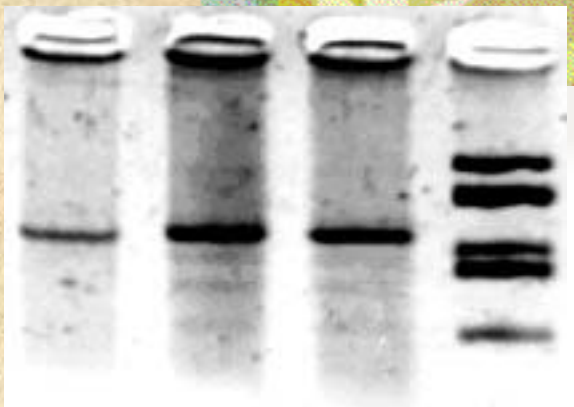
- **Heterozygosity on investigated genetic-biochemical markers in carriers of BLAD mutations and animals free of mutations in economies “Askania-Nova” and “Knyazhichi”**

Economies	“Askania-Nova ”		“Knyazhichi”	
Loci	Animals free of BLAD	BLAD	Animals free of BLAD	BLAD
TF	0.591	0.632	0.731	0.632
AM	0.576	0.474	0.440*	0.600*
CP	0.409*	0.526*	0.680	0.600
PN	0.087***	0.286***	0.150**	0.356**
GC	0.106**	0.308**	0.400**	0.600**
PTF	0.258**	0.615**	0.560	0.400

Note: * - P < 0,05; ** - P < 0,01; *** - P < 0,001

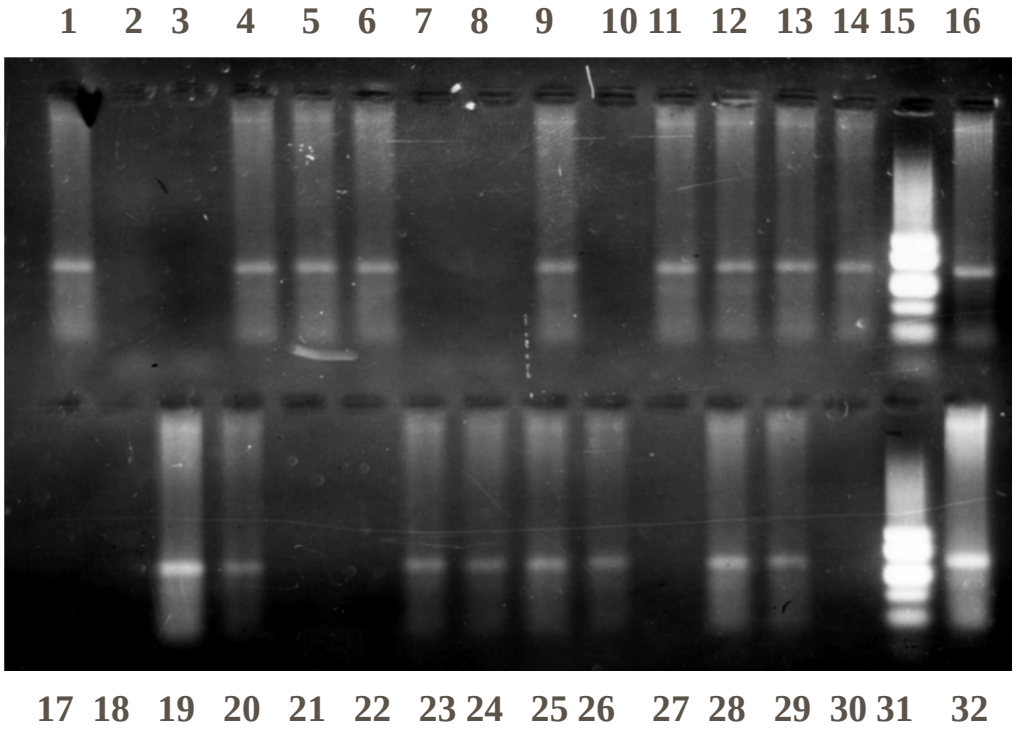
- **Results of the carried out comparisons of associations between heterozygosity on the number of genetic-biochemical systems and heterozygosity on loci CD18 and CSN3 testified that fast distribution of mutation BLAD (in locus CD18) could be caused by the influence of the natural selection supporting heterozygosity on different loci at the introduction of animals in new environment conditions of breeding in a greater degree, than selection on the raised nonspecific resistance to various pathogens in which take part both locus CSN3, and locus CD18.**

- **Revealing of infectious agents, for example, the provirus of Bovine Leucosis Virus (BLV) in cattle genomes**



Selection of PCR optimum conditions with primers to virus gene env. It was used DNA allocated from cell culture of embryonic kidneys of lambs (FLK), infected BLV. Electrophoretical division of products of amplification in 3 % agarose: marker of molecular mass - Puc 19, restricted by Hae III (line 4, from left to right); a product of amplification of gene env – lines 1-3.

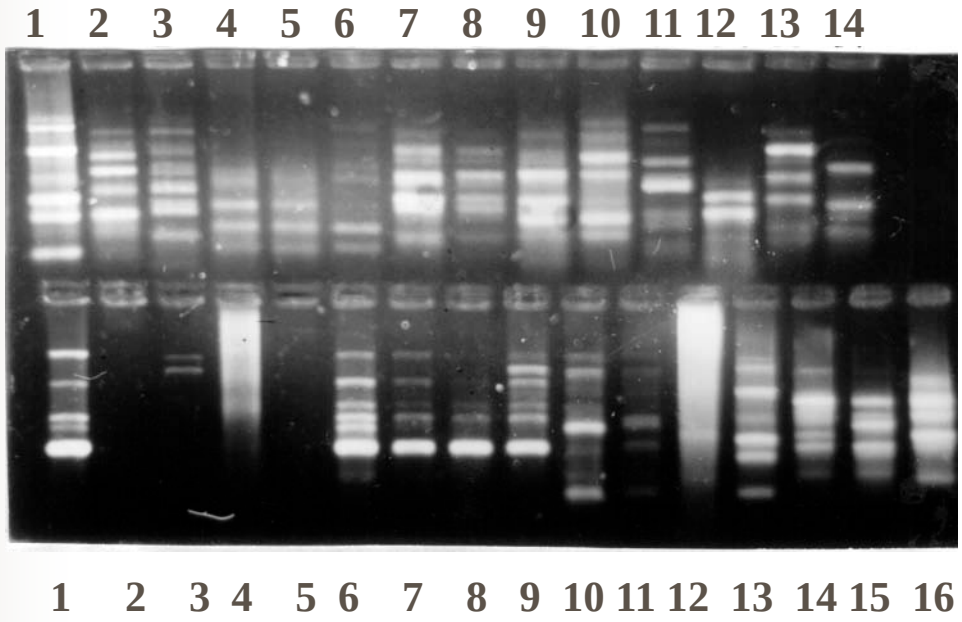
Testing of cattle on presence of provirus DNA BLV in genome by the PCR using with the primers to provirus gene env. Electrophoretical division of amplification products in 1.5 % agarose: the marker of molecular mass Puc 19 – lines 15, 31 (from left to right); the animals investigated on the presence of DNA provirus in genome – lines 1-14; 17-29; product PCR received on DNA from culture of infected FLK cells – lines 16, 32.



Another way of DNA method using is the investigation of conservatism and polymorphism of different genome elements – to understand the rule of genome organization and variability and, may be, the gene network organization.

- **Unlike point mutations and segment duplications, the analysis of distribution of the invert repeats allows to estimate features of mutual positioning of nucleotide motives possessing the ability to cointeract in one-chained DNA and, thus, capable to participation in formation of DNA secondary structures of DNA, necessary, in particular, for the identification of regulatory signals.**

RAPD-PCR primers UBC-85, (top) and UBC-126, (below) in different Ungulate species



1-14 UBC-85: 1–Arabian horse; 2 – Grey Ukrainian cattle; 3 – Bison bison; 4-6 – Blue buck; 7,8 – Zebra Grevi; 9,10 – Donkey; 11- pig; 12 – snow buck; 13–kulan; 14 – saiga.

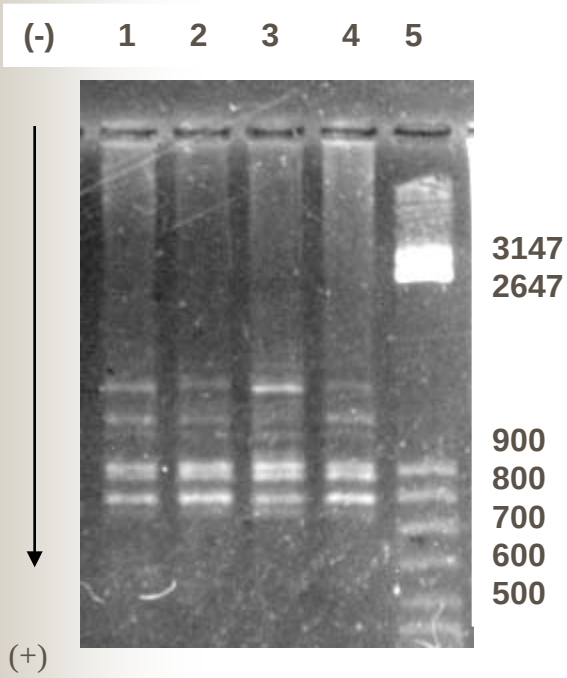
1-12 UBC-126: 1–Arabian horses; 2 – Bison bison; 4-5 – Blue buck; 6,7 – Zebra Grevi; 8,9 – Donkey; 10,11- eland; 12 – Antelope Gnu;
13-16 UBC-85: 13 – Arabian horses, 14,15 – eland; 16 –Antelope Gnu

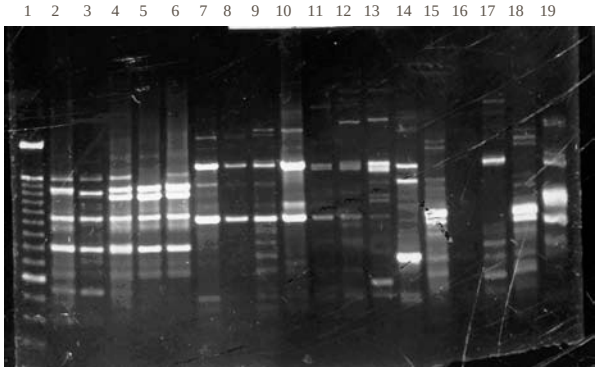
- **Amplification of DNA fragments, flanking by decanucleotides, depends from their nucleotide sequences;**
- **distribution of inverted decanucleotide repeats in some species had the expressed taxa-specific features;**
- **spectra of amplification products, basically, were defined by 6 nucleotides on 3' end.**

- **Distribution of inverted repeats of decanucleotides UBC-85 and UBC-126 is not random - for such taxa as human, rodents, other mammals, vertebrates and plants some amplicons of the same lengths were “gain" in comparison with others.**
- **The potential amplicons in invertebrates, viruses and prokaryotes were comparative more regular. Nevertheless, the spectrum of potential amplicons in sequences of viruses, received with use of UBC-85 (252 amplicons), essentially differs from revealed with the use of UBC-126 (38 amplicons).**
- **That is, obtained data testified to presence of the certain nonrandomness of distribution of inverted decanucleotide repeats in relation of specificity of decanucleotide motives and investigated taxa**

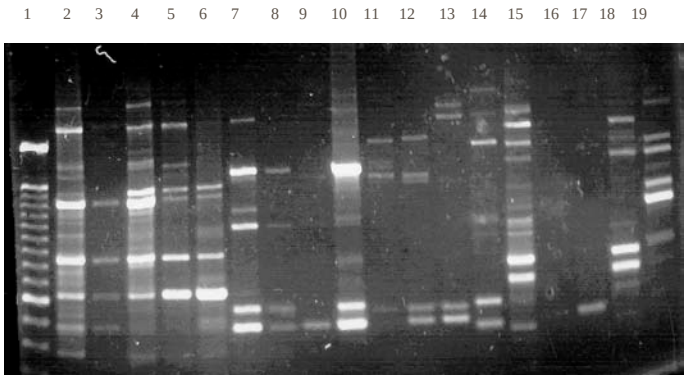
The examples of amplification products of DNA fragments, flanking by microsatellite loci (ISSR-PCR)

Sheep - primer (AGC)6G



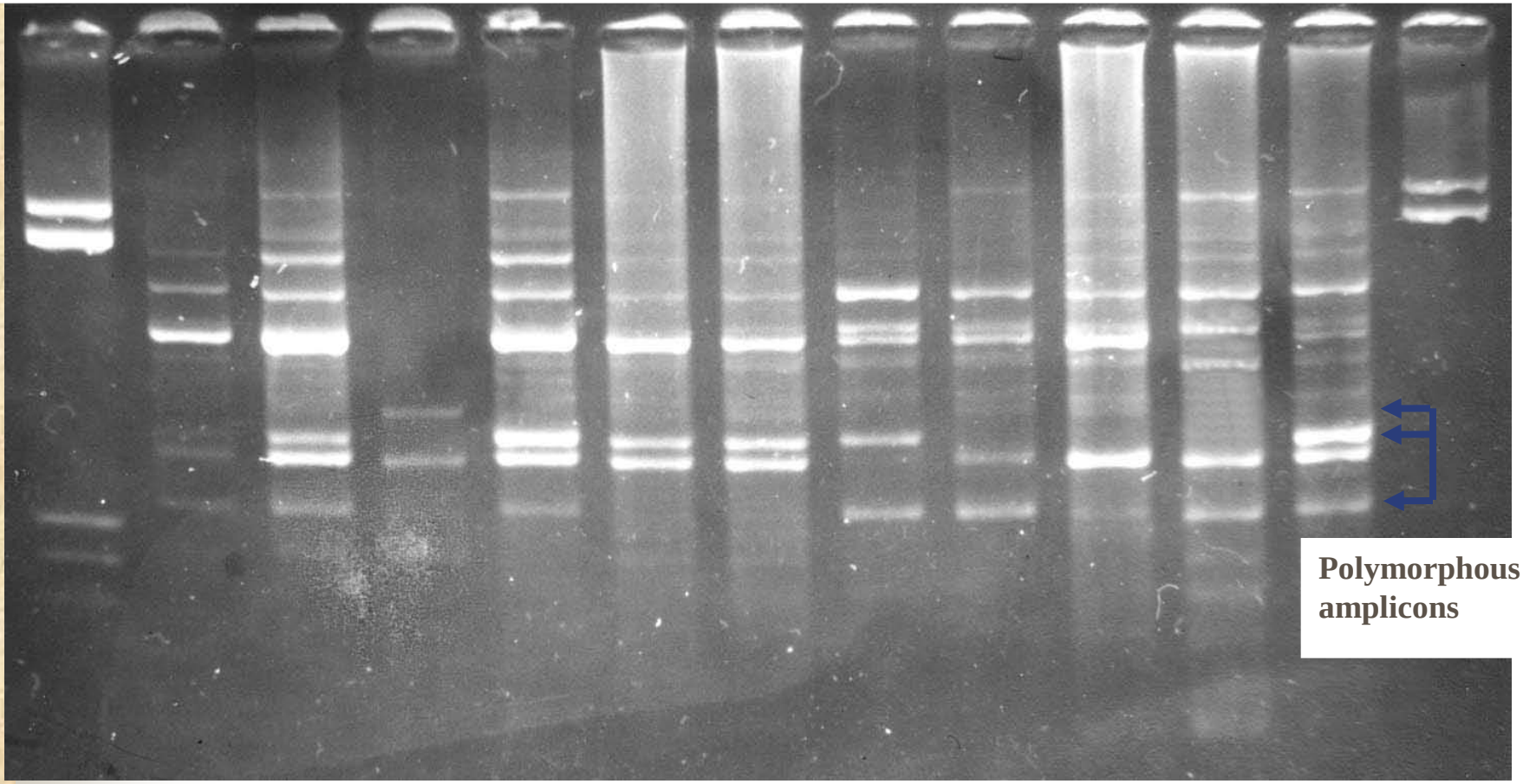


Amplicon's spectra of *Ungulate* species with primer (AG)9C: 1– marker of molecular weights; 2 – domesticated horse; 3 – Przewalsky Horse; 4 – kulan; 5 – Zebra Grevi; 6 – Zebra Chapman; 7 – cattle; 8 – Bison bonasus; 9 – Bison bison; 10 – Bos gaurus; 11 – sheep; 12 – snow buck; 13 – Ammotragus lervia; 14 – saiga; 15 – Antelope Kanna; 16 – Antelope Nilgau; 17 – Antelope Gnu; 18 Antelope Garna; 19 – pig

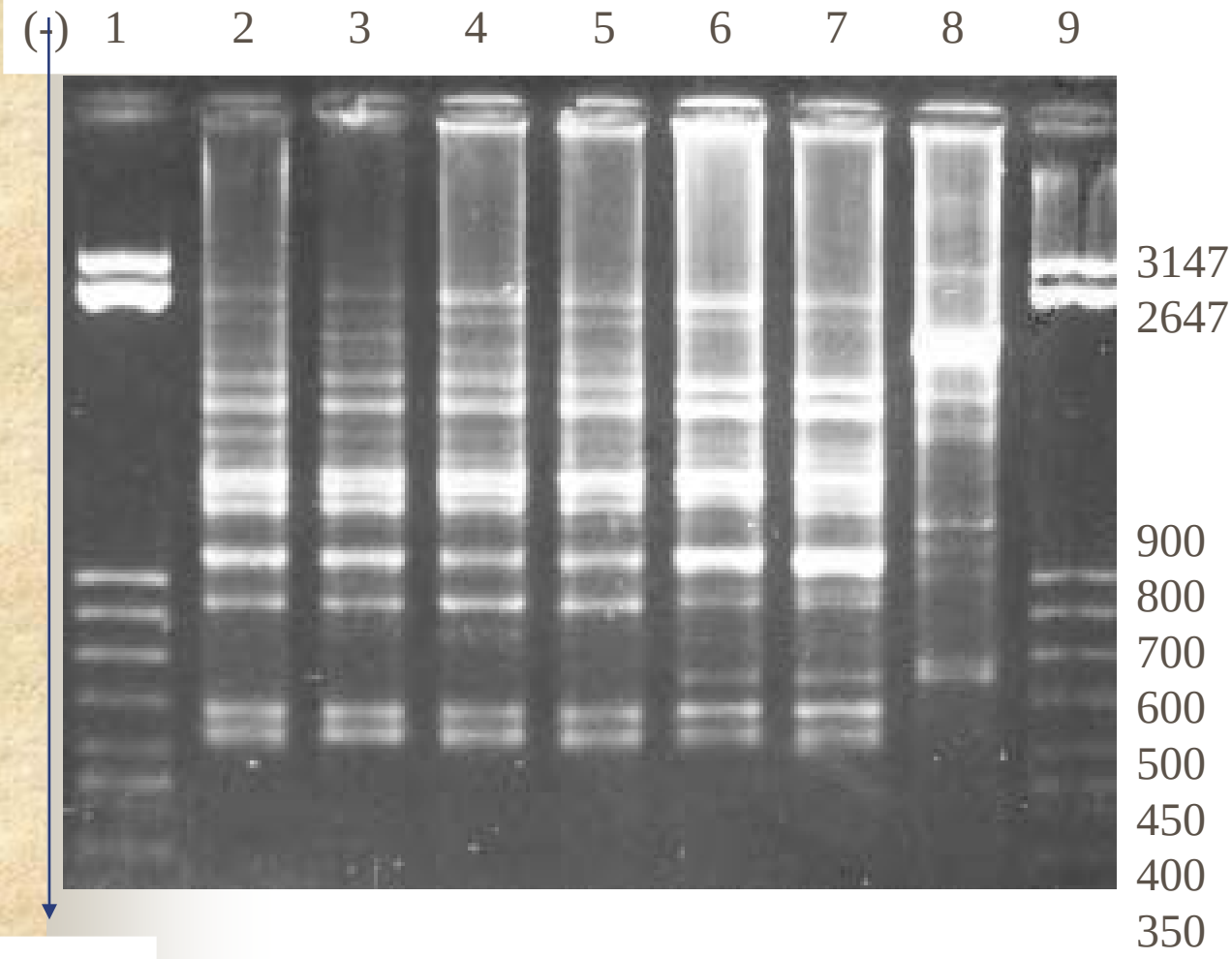


Amplicon's spectra of *Ungulate* species with primer (GA)9C: 1– marker of molecular weights; 2 – domestic horses; 3 – Przewalsky Horse; 4 – kulan; 5 – Zebra Grevi; 6 – Zebra Chapman; 7 – cattle; 8 – Bison bonasus; 9 – Bison bison; 10 – Bos gaurus; 11 – sheep; 12 – snow buck; 13 – Ammotragus lervia; 14 – Antelope saiga; 15 – Antelope Kanna; 16 – Antelope Nilgau; 17 – Antelope Gnu; 18 – Antelope Garna; 19 – pig.

The example of spectra of amplification products ISSR-PCR (primer (CTC)6G) on DNA of Grey Ukrainian cattle.



The example of spectra of amplification products ISSR-PCR (primer (ACC)6G) on DNA of Grey Ukrainian cattle.



Lines 1,9 – marker of molecular weight;
Lines 2-5 – amplicon spectra of DNA of cows;
Lines 6-8 – amplicon spectra of DNA of bulls.

- **Spectra of DNA fragments, flanking by the inverted microsatellite repeats with the same core motive, but with different "anchor" nucleotide on the 3 ' the end, essentially differ from each other, that testifies of the precisely of method.**
- **Spectra of amplification products of such fragments essentially depend from nucleotide sequences of primers.**

Comparative analysis of the lengths of the amplification products in domesticated and wild species of Ungulate, obtaining with the use as primers decanucleotides (RAPD-PCR) and fragments of microsatellite loci (ISSR-PCR)

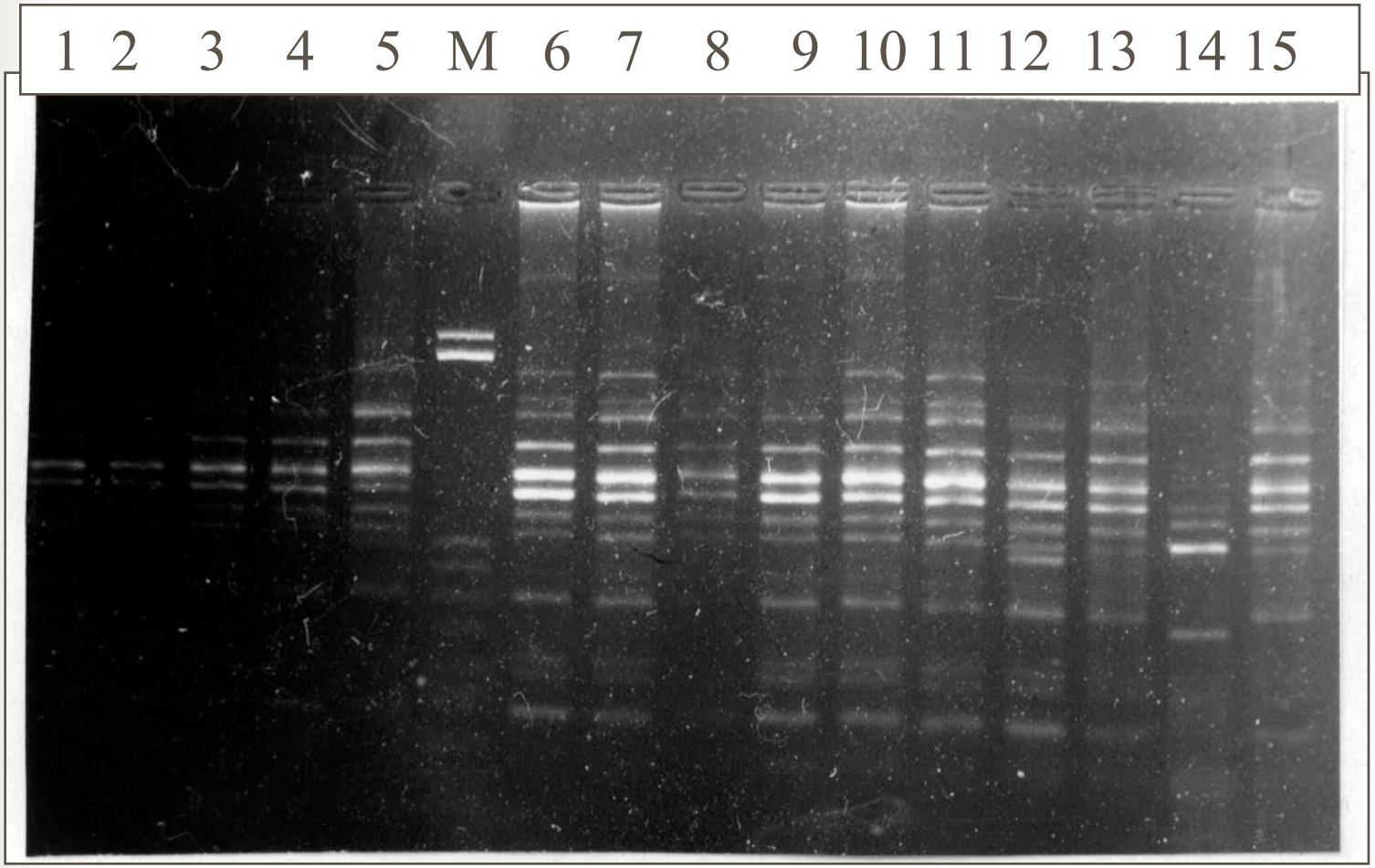
Species	Amplicon's lengths		
	short (400-1000 bp,%)	middle (1100-1900 bp, %)	long (2000-2500 bp,%)
ISSR-PCR			
Domestic	46,7	43,0	10,3
Wild	40,7	43,7	15,6
Dolphins	60,0	36,0	4,0
RAPD-PCR			
Domestic	36,3	50,9	12,8
Wild	29,8	49,0	21,2

In investigated wild species, in general, it was revealed more long DNA fragments, flanking by invert repeats or decanucleotides, or microsatellite locus in comparison with close related domestic species. The short fragments were observed more often in genomes of domesticated species.

- **The greatest quantity of amplification products was received with the use as primers of the fragments the purine/pyrimidine sequences (three nucleotide microsatellites GAG, CTC).**
- **For DNA fragments, flanking by such inverted repeats, marked also the greatest conservatism on lengths of the amplification products, received on DNA genomes of various mammalian species.**
- **Obtained data allowed to assume a special role of such inverted repeats in super nucleotide level of the organization of a genetic material**

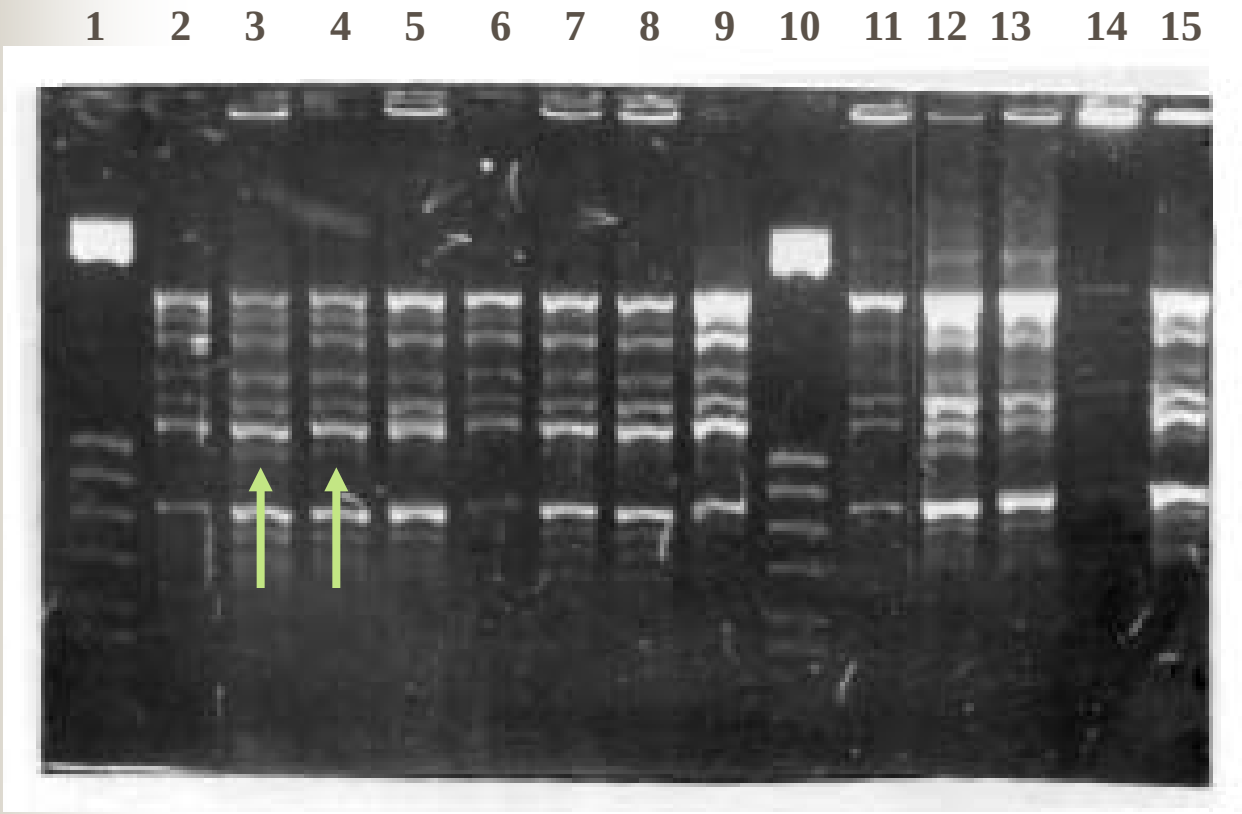
Polymorphism of DNA fragments, flanking by invert repeat of part of transposone LTR SIRE-1 (N AF053008).

Lines 1-4, 5-10 – DNA of cattle from the experimental herd «Novoshepelichi»; lines 11-15 – DNA from control group of cattle from "pure" zones, M – marker of molecular weights.



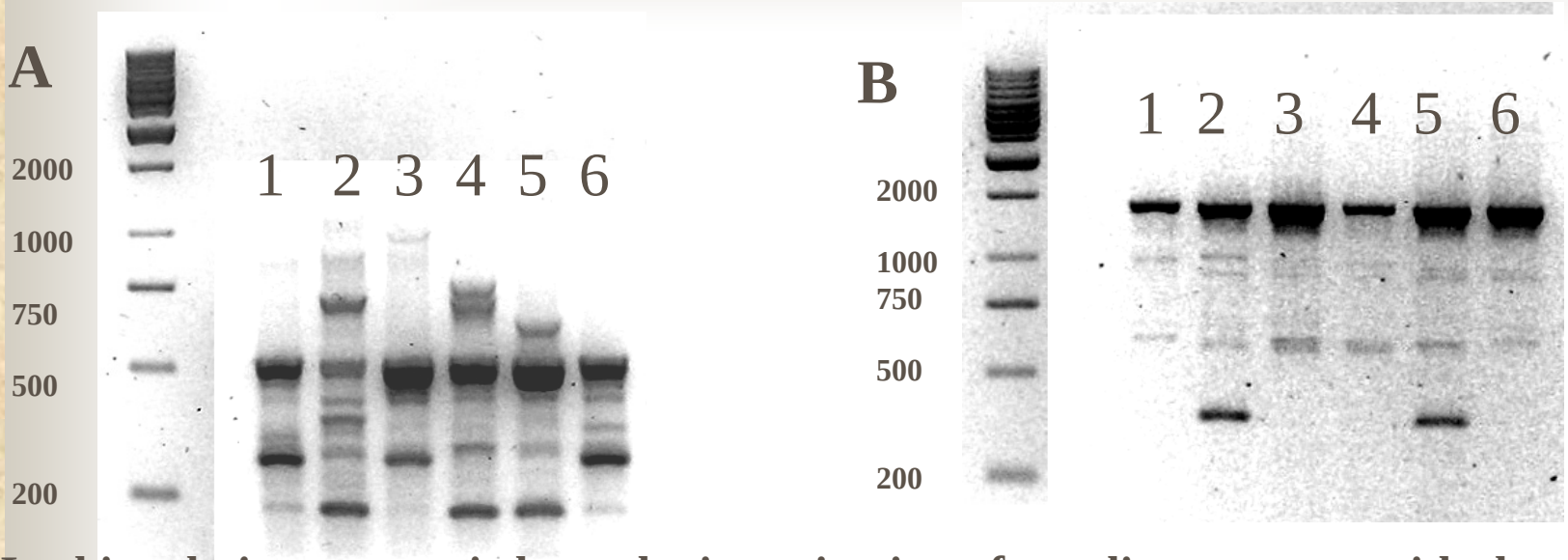
The tissue-specific polymorphism of amplification products, revealed with the use as primer the flanking fragment of transposone LTR SIRE-1 in *Microtus oeconomus* from Red Forest

Red arrows marked the amplification products, which presented in muscle and liver (lines 3 and 4) but absented in kidney (line 2) of the same animal.



- **The analysis of distribution of DNA fragment lengths, flanking by the inverted repeats of terminal retrotransposone sequences in rice varieties also testified the absence of the equiprobable dispersion of such fragments on genome length.**

Accumulated data allowed assuming that most polymorphous variant of molecular-genetic markers for resolving of number of applied tasks in rice breeding could be the DNA fragments, associated with the transpose elements.



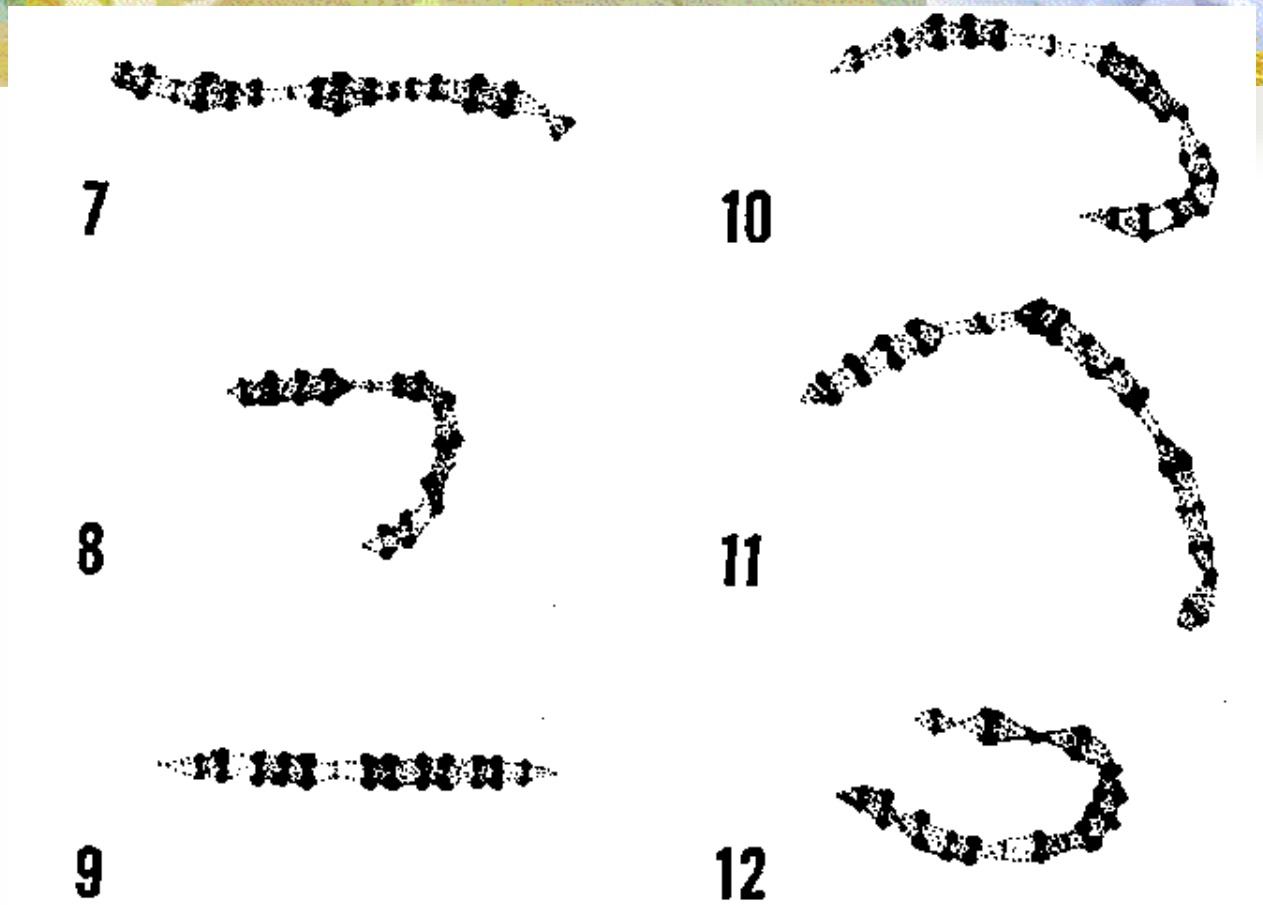
In this relation we carried out the investigation of amplicon spectra with the use as primers in PCR of DNA fragments, flanking by terminal repeats of retrotransposons belonging to family R 173 (A - S5, B - S6).

The gene pools of 6 rice varieties – Jantar (1), Dalnevostochnii (2), Fontan (3), Lider (4), Liman (5) and Primorskii (6) - were analyzed. The each variety had the variety-specific combination of amplification products with the DNA fragment lengths from 1000 to 200 b.p. It is shown the high level of polymorphism of site of retrotransposon integration and the high productivity of using of DNA fragments, belonging to transposon sequences, as molecular genetic markers for variety identification in rice.

Obtained data testify the certain intra genome organization of the inverted repeat distribution with separate nucleotide motives on their flanks.

Such organization will be coordinated well with the hypothesis of Lima de Faria about the nonrandomness of alternation heterochromatic blocks on chromosome length of some plant species.

He formulated the hypothesis about "chromosomal fields", owing to which nucleotide sequences and the congestion of various families of repeats, including centromere and telomere repeats, close realted with the chromosome morphology.



Nonrandomness distribution of chromomeres in meiotic chromosomes between centromere and telomeres, illustration of Lima de Faria for his theory about “chromosome landscapes” and “chromosome phenotypes”

- **The obtained data visually show the possibilities of the using of short DNA fragments (20 - 70 nanometers) for in-depth studies of genomic organization in nano-meter scale and genetic-biochemical mechanisms of cellular and tissue phenotype formation and to develop the experimental approaches to its prognosis and correction.**