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„THESIS OF DOCTORAL (PHD) DISSERTATION"

Estimation of genetic distance among Tsigai and Zackel sheep population and expression and polymorphism study of three non-classical immungenes in pig

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I. INTRODUCTION

In my thesis I want to display my researches I performed on two domain with two varieties of domesticated animal species. One is the determination of the genetic distance of - sheep flock belonging- Tsigai and Zackel using microsatellite markers. The other falls under the discipline of immunogenetic. This is the research of expression and polimorphism of non classical MHC I genes in pig. The two subjects seem to be far from one another, but it can demonstrate the possibility of wide adoption of biotechnological researches.

Hungary is one of the first countries where is recognized the preservation of domestic animal species, the conservation of the entire genetic variancy, and the regulation of function and relation of different genotypes is a very important task in cultural and technical aspects too. In Hungary the Racka, Tsigai and Cikta cultures were almost entirely liquidated in the previous decades, and the headcount of Racka and Tsigai flocks began to grow only in the last decade. As the support of biological diversity came to the front, and with the environmental agriculture their breeding became general, these races are the most optimal for extensive keeping (OLÁH, 2002). Nowdays we do not know, that in these species what kind of genes, and with what frequency occured, we only suppose, that there were more genes than today, so we have to hinder the loss of further genes too (FÉSÜS, 1997).
The Tsigai got into Hungary around 1700. The claim of the blaize-factories inspired the farmers in Transylvania to change the rough woolly Curkan to Tsigai, which produce more softer wool (RODICZKY, 1904; cit.: GÁSPÁRDY, 2002). Tsigai as a new variety was displayed for the professional public on the millennial animal-exhibition in Budapest in 1896 (GÁSPÁRDY, 2001). Many Tsigai variation is bred in East-and Central European countries and other regions. These variations differ in bodysize, bodyweight, growth and colour too. In Great Serbiania on the region of Bácska and Bánáti the more extensive Cokanski and the heavier, better milking Zomborsky type is bred. The home representative of the latter is the Lédeci of sorts flock (DUNKA, 1997). The Transylvanian Tsigai has more chest width, relatively shorter legs. Covasna variant has brown head and legs (GÁSPÁRDY, 2001). In Bulgaria it has also two types, the North-West and the South-Bulgarian one. (DIMOV, 2000; cit.: KUKOVICS és JÁVOR, 2002). Nowdays we distinguish two types of Tsigai in Hungary, the

autochthonous and the –selected for milk production- Zomborski variant. Between these there are several transitional types (KUKOVICS et al, 2003). Earlier the blood type and protein polimorphism was examined to demonstrate genetic distinctions (FÉSÜS, 1974). Nowadays the usage of genetic markers became general to estimate genetic distance, because it is more efficient and has more sureness. Researches which are based on DNA microsatellitee markers -these are easier and faster- afford more particular informations about the determination of genetic distinctions among breeds (BARKER et al, 1997; MACHUGH et al, 1997).

The aim of biotechnological and genetic examinations can be the xenotransplantation too. The idea of transplantation from animal to human, interests mankind for a long time. As the size of the organ of pig is similar to human's, and has less mutual pathogen with human than by primates, so this is the most searched kind. The cellsurface identifiers of a transplanted animal organ or tissue are enemies of the receiver immue system, so the defense starts up immediately. The histocompatible antigenes determine whether the animal transplant or tissue is immunologicaly compatible with the receiver or no. If there is a large difference, histocompatible antigenes come into action, which create rejection immediately, (the antigenes, which encode these genes can be found in the MHC (major histocompatibility complex) region). In absolute absence of MHC genes there is no human illness, because the proteins which are encoded by genes are so important that the fetus perishes in the lack of it. It is observed a long time ago, that some alleles of the classical MHC genes are in connection with the responsiveness of many illnesses, nowdays more than 5000 illnesses are found in connection with this (malaria, tuberculosis, dengue-fever, hepatitis, AIDS) (TROWSDALE, 2005; TRAHERNE et al, 2006). In case of MHC we distinguish three kind of domains, the MHC I, MHC II és MHC III. The MHC I molecule can be found on almost each cellsurface of the organism, these are responible for the MHC I glicoprotein synthesis. These can be devided into two more groups, classical MHC I genes (Ia) and non classical MHC I genes (Ib). In human and pig can be found also three genes in the group of classical and non classical group of MHC I genes. In the examination of classical genes these are HLA (Human Leucocyte Antigen) –A, -B and –C in human and SLA (Pig Leucocyte Antigen) -1, -2 and -3 in pig, while the non classical genes are HLA –E, -F and –G in human and SLA -6, -7 and -8 in pig. HLA genes can be found on the sixth chromosome, while SLA genes are on the seventh chromosome. The classical

Ia genes present the peptids to the CD 8+ limphocytes, these are polimorphic and they have no tissue specific expression. They have conserved structure (SIMOND et al, 2005). The non classical MHC I genes are not so polymorphic, they have tissue specific expression in human and the all gene exact function is not known (CLEMENTS et al, 2005; KNAPP et al, 1998). Among human Ib genes we have the most information about HLA-G. The HLA-G gene has an important role in forming of immunotolerance during pregnancy (CAROSELLA, 2005). It is essential of uncluttered procession of pregnancy, that on optimal immunalogical environment evolves in the body of the mother. For the mother the fetus is an immunologically foreign population, so the fetal antigenes of immune system of the mother must be recognized, wherein the HLA-G gene plays an important part. (SZEKERES-BARTHÓ, 2005).

There is no ortological relation within MHC Ib genes between human and pig neither in their sequence nor in their position (CHARDON et al, 2000).

According to CREW et al (2004) SLA-6, -7,-8 genes have tissue specific expression. 11 different samples were examined by reverse-transcriptase PCR method (brain, heart, kidneys, liver, lung, muscle, spleen, testis, thymus, peripheral blood mononuclear cells). According to their results SLA-6 was expressed in each sample, the SLA-8 expressed in each except the brain, the expression of SLA-7 gene was mostly tissue specific. According to others' results expression of SLA-8 was the strongest in the placenta and probably this is the homolog of HLA-G. (SMITH et al, 2005). To judge by the measure of expression of SLA-6 in the spleen, it can be similar to HLA-E gene, but it can not be stated, because other functional examinations are needed (KOLLER et al, 1998).

SIMOND et al (2005) examined the polimorphism of 8 locus in the Westran and in Large White pig. In SLA-1, -2, -3, -6 locus new alleles were identified, but the locus SLA-6 showed low degree of polymorphism. To examine exon 2, 3 also a low degree of polimorphism was found in Yucata breed (SMITH et al, 2005).

II. AIMS OF THE RESEARCH

The aims of genetic distance estimation part of my thesis were:

1. the selection of microsatellitee markers, whereby the examination of genetic distance estimate in Tsigai and Zackel population is can be easily executed;
2. to determine the genetic variability with these microsatellitee markers, genetic relationship and differences among examined population;
3. to compare our results with the results of other examinations;
4. to define whether the geographical separation is parallel with genetic separation.

We had the following aims in immunogenetic part of thesis:

1. to design gene specific and effectively usable primers for gene expression and polimorphism study;
2. to study the expression of MHC Ib genes in case of different sex and age pig on mRNA level;
3. polymorphism study of MHC Ib genes in different pig breeds.

III. MATERIALS AND METHODS

<u>Study of genetic difference</u>

Our research was supported by European Regional Focal Point for Animal Genetic Resources project, titled „Possible way of conservation the multipurpose Tsigai and other indigenous sheep breeds in Central-, Eastern and Balkan countries”. This program was coordinated by Hungary and Albania, and its supervisors (Dr. Sándor Kukovics, Kr. Kristaq Kume) organized the collection of samples from different countries. Our research was done from blood sample of Pál Gábor’(selfemployed person) Milking Zomborski, Transylvanian Rusty and from the Farm of University Debrecen Centre of Agriculture Sciences Department of Animal Breeding and Nutrition Cokanski Tsigai flock (n=252), while all the other case we had hair samples (n=1253) *Collecting of the hair and blood samples* were started in 2004, because the sampling was numerous and included 8 (Hungary, Romania, Albania, Bulgaria, Croatia, Turkey, Slovakia, Serbia-Montenegro) countries and it required a long preliminary preparation (Table 1.).
The *blood sample was taking* from animals’ vena jugularis (2,5-3,0 ml per individual) and immediately got into special tubes which were containing anticoagulant. The samples were stored on -20°C till next step. The *isolation of genomial DNA from blood* was done following the ZSOLNAI and ORBÁN (1999) method. The *taking of hair samples* was done by picking and the samples were taken into nylon or paper bags. The *extraction of the genomial DNA* was followed the worked out method of FAO (2004). The genomial DNA samples were stored on -20°C for further steps.

The examinations were done in molecular genetic laboratory of UD-CAS Department of Animal Breeding and Nutrition and Agricultural Biotechnology Centre in Gödöllő.

In Table 1. the examined flocks are presented, their numbers per population and their label which were used during the research.

Table 1: Used population and their main character

Country	Population	Group	Number	Label
Hungary	Autochthonous	Tsigai	53	HU-SMA-AC
			40	HU-KMKK-AC
			39	HU-KMNP-AC
			53	HU-SZIC-AC
			45	HU-MRD-TAC
	Cokanski	Tsigai	125	HU-DE-CSC
	Zomborski	Tsigai	77	HU-PG-ZC
			39	HU-LB-TCZ
			42	HU-OJ-TC
	Rusty (from Jucu)	Tsigai	50	HU-PG-TRC
Rumania	Romanian Ruda		40	RO-RUDA
	Rusty (from Turda)	Tsigai	40	RO-RUST-TS
Albania	Albanian Tsigai	Tsigai	39	AL-TS
	Albanian Ruda	Zackel	37	AL-RUDA
	Bardhoke	Zackel	31	AL-BARDH
Bulgaria	Patched Faced Maritza	Zackel	39	BU-PFMAR
	Pleveny Blackhead sheep	Zackel	35	BU-PLBH
	Rodopski Tsigai	Tsigai	30	BU-ROD-TS
	Staroplaninski Tsigai	Tsigai	42	BU-STAR-TS
	White Maritza sheep	Zackel	41	BU-WFMAR
Croatia	Croatian Tsigai	Tsigai	50	CR-TS
Turkey	Sakiz	Zackel	49	TR-SAKIZ
	Gokceada	Zackel	42	TR-GOKCE
	Kivircik (Marmara region)	Tsigai	46	TR-KIV-MAR
	Kivircik (Trakya region)	Tsigai	53	TR-KIV-TRA
Slovakia	Handel	Tsigai	25	SL-HAN-TS
	Jugat	Tsigai	22	SL-JUG-TS
	Kamo	Tsigai	19	SL-KAO-TS
	Sirig	Tsigai	22	SL-SIR-TS
	Vojin	Tsigai	5	SL-VOJN-TS
	Jurbis	Tsigai	24	SL-JUR-TS
	Kamendin	Tsigai	16	SL-KAM-TS
	Olymp	Tsigai	5	SL-OLYM-TS
	Ondrej	Tsigai	16	SL-OND-TS
	Rybar	Tsigai	16	SL-RYB-TS
	Vancover	Tsigai	15	SL-VAN-TS
	Brend	Tsigai	10	SL-BREN-TS
Serbia-Montenegro	Zomborski	Tsigai	41	SM-ZP-TS
	Cokanski	Tsigai	12	SM-CS-TS
	Svrljiska Zackel Pramenka	Zackel	48	SM-SVR-PR
	Krivovirska Zackel Pramenka	Zackel	32	SM-KRI-PR

Our research were done with the following *microsatellites*:
BM 6506 (1); OarFCB 20 (2); MAF 70 (4); MCM 527 (5); INRA 127 (8); ILSTS 11 (9); TGLA 53 (12); TGLA 357 (14); MAF 65 (15); Oar CP 49 (17); Oar AE 119 (19); Oar CP 20 (21); BM 1314 (22); MAF 35 (23); MCMA 7 (25); CSSM 43 (26).
We used ABI 9700, ABI2700 and MJ Research Thermocycler programable PCR (DNA Thermal Cycler) for *PCR reactions*. The *detection and examination of alleles* were done with ABIPRISM 3100 Genetic Analyzer. The *collection of data* was done by the help of GeneScan software (Applied Biosystems). The *evaluation of informations* was done by Genographer software. In the course of evaluation of *statistic data* we used the POPULATIONS, GENEPOP, MICROSAT, PHYLIP and ARLEQUIN version 2.0 softwares.

Study of gene expression

The following *samples* were collected *from adult* sow and boar:
longissimus dorsi muscle, ham muscle, aorta, heart, diaphragm, spleen, thymus, uterus, kidney, ovary, testicle, peyer plaque, epididium, ileum, duodenum, jejunum, pancreas, liver, lung, skin, ganglion ainguinal, adrenal glands, brachial nerve, tonsil, brain, sinew, ear cartilage, foot cartilage, nose cartilage, nose mucous, tail fat, neck fat, back fat.
Samples of *100 days old fetuses* are the following:
Longissimus dorsi muscle, ham muscle, aorta, heart, diaphragm, spleen, thymus, kidney, ovary, testicle, epididium, ileum, jejunum, duodenum, pancreas, lung, skin, ganglion ainguinal, brain, nose cartilage, foot cartilage, ear cartilage, umbilical cord, placenta

Total RNA isolation was done with QIAGEN EASY Lipid, Fibrous, Classical kits (QIAGEN, France) according to the type of tissue. The quantity and quality of the total RNA was tested in all case. These were stored on -80°C.
Primer Express and Primer 3 program and manually were *designed primers*. In all case we designed the forward and reverse primers into exon 2, 3 and junction. We have chosen 1 primer pair per gene after checking its amplification and gene specification.
During the *absolute quantification* we determined the best primer concentrations, the efficiency of PCR, standard curves of all examined genes and the best cDNA concetration. The reactions were fulfiled using ABI 7900 and SDS 2.1. software.
During *relative quntification* we used the $\Delta\Delta$Ct method.

Study of polymorphism

For *genomic DNA isolation* Gensiol Maxi Prep kit (ABgene, United Kingdom) was used following the orders of the manufacturer. DNAs were stored on 4°C till utilization. Different *European and Chinese pig breeds* were used (Table 2.).

Table 2: Used breed for polymorphism of SLA Ib genes

Origin	Examined breed	Identification number
Europe	Large White	E1
	dd (H04/H04)	369
Melim program (Melanoma bearing Libechov minipig)		
1st cross	Melim	60143
	Duroc	51176
	F1 (MelimXDuroc)	81015
	F1 (MelimXDuroc)	81016
2nd cross	Melim	310
	Duroc	270
	F1 (MelimXDuroc)	79
	Duroc	10831
	BC (F1XDuroc)	31044
	BC (F1XDuroc)	31045
China	Guizou	4
	Dawei	2
	Bama	3
	Meishan	1
	Erhua lian	2
	Xiang	1
	Laiwu	2
	Yimeng	1
	Min	1
	Lantang	2
	Wuzhi shan	2

For this research our aim was to amplify the whole exon 2. We checked the gene specificity of designed primer. After *cleaning PCR products and determing their sequence* we used NovoSNP 2.0.3. program (WECKX et al, 2005).

IV. MAIN RESULTS OF THE THESIS

The results of estimated genetic distance of Tsigai and Zackel sheep flocks

The genetic distance research, which covered 41 Tsigai and Zackel flock in 8 countries, was fulfiled by the help of microsatellite markers. On the examined locus we determined totally 384 alleles. The least number of alleles (11) were found on locus MAF35, the most larger number of alleles (35) were found on locus MAF70. The avarage number of alleles is ranged between 4.1 (MAF35) and 10.4 (MAF70) (Table 3.).

The inbreeding, the long breeding without blood refreshing can reduce the level of genetic variability and gives low value in the aspect of heterozygous, specially in the case of small number populations.

The expected mean value of heterozygosity was on the 16 locus 0,716. The least number was (0,614) in BM6506, the most higher value was (0,812) on locus BM1314. In case of every markers the expected heterozygosity value was higher than the observed value. The mean observed heterozygosity value was 0,525. We have got the least number in case of MAF65 (0,3190), the largest one in case of locus BM1314.

The mean heterozygosity value among examined population was between 0,356-0,629, the expected value is changing between 0,640-0,843. All examined populations were less heterozygous than it was expected. The absence of heterozygous individuals were the largest in the Serbian Zomborski population (SM-ZP-TS), the least in one of the Hungarian autochthonous population (HU-SMA-AC). According to inbreeding coefficient the inbreeding failure is higher in the Serbian Zomborski population (52,7%), least in Croatian Tsigai population (12,8%) among examined populations. Based on the mean number of alleles, the Hungarian Cokanski population, HU-DE-CSC (8.8) is the most diverse, the least diverse population is the Serbian Zomborski, SM-ZP-TS (2.3) (Table 4.).

Table 3: Main character of examined locus

Locus	Length (bp)	Number of detected allele	Mean number of allele per locus	H_{obs}	H_{exp}	F_{is}
MAF35	104-122	11	4,1	0,494	0,623	0,207
CSSM43	237-273	26	8,7	0,509	0,795	0,361
MCM527	150-185	20	7,1	0,540	0,750	0,281
TGLA53	114-143	24	8,2	0,634	0,788	0,196
MCMA7	228-270	31	8,0	0,622	0,750	0,171
OarFCB20	92-118	22	6,6	0,407	0,687	0,408
TGLA357	113-154	27	7,9	0,584	0,767	0,238
INRA127	181-215	31	6,2	0,605	0,677	0,106
MAF70	128-175	35	10,4	0,423	0,794	0,468
MAF65	116-140	18	5,6	0,319	0,674	0,526
ILSTS11	180-296	23	6,1	0,600	0,713	0,158
OarCP20	88-195	17	5,1	0,500	0,677	0,262
OarCP49	88-140	28	5,6	0,619	0,681	0,092
BM1314	136-176	32	8,6	0,644	0,812	0,207
BM6506	184-212	21	5,0	0,554	0,614	0,097
OarAE119	98-160	18	4,7	0,352	0,645	0,455
Mean		24	6,8	0,525	0,716	0,264

Table 4: Number of detected allele, heterozygous values and estimated inbreeding values of examined populations

Populations	Number of detected allele	Mean number of allele per population	H_{obs}	H_{exp}	F_{is}
HU-DE-CS	140	8,8	0,518	0,746	0,305
HU-PG-ZC	130	8,1	0,500	0,759	0,341
HU-PG-TRC	130	8,1	0,594	0,766	0,225
HU-LB-TCZ	115	7,2	0,552	0,694	0,205
HU-SMA-AC	126	7,9	0,629	0,745	0,156
HU-OJ-TC	129	8,1	0,613	0,771	0,205
HU-KMKK-AC	129	8,1	0,597	0,770	0,225
HU-KMNP-AC	107	6,7	0,584	0,718	0,192
HU-SZIC-AC	118	7,4	0,542	0,767	0,291
HU-MRD-TAC	106	6,6	0,506	0,724	0,299
AL-TS	130	8,1	0,528	0,739	0,285
AL-RUDA	117	7,3	0,500	0,761	0,343
AL-BARDH	107	6,7	0,525	0,747	0,297
CR-TS	107	6,7	0,594	0,682	0,128
TR-SAKIZ	95	5,9	0,436	0,640	0,319
TR-KIV-MAR	100	6,3	0,486	0,678	0,282
TR-KIV-TRA	130	8,1	0,483	0,772	0,374

TR-GOKCE	100	6,3	0,503	0,772	0,349
RO-RUST-TS	128	8,0	0,577	0,787	0,267
RO-RUDA	118	7,4	0,504	0,768	0,343
SM-ZP-TS	36	2,3	0,356	0,753	0,527
SM-CS-TS	85	5,3	0,512	0,765	0,330
SM-SVR-PR	91	5,7	0,392	0,716	0,452
SM-KRI-PR	98	6,1	0,416	0,728	0,428
BU-STAR-TS	115	7,2	0,458	0,726	0,366
BU-ROD-TS	100	6,3	0,520	0,735	0,290
BU-PLBH	121	7,6	0,490	0,798	0,376
BU-PFMAR	124	7,8	0,577	0,769	0,246
BU-WFMAR	138	8,6	0,568	0,787	0,275
SL-OND-TS	112	7,0	0,574	0,835	0,313
SL-KAM-TS	104	6,5	0,552	0,806	0,315
SL-KAO-TS	110	6,9	0,461	0,751	0,386
SL-SIR-TS	127	7,9	0,487	0,805	0,395
SL-VAN-TS	79	4,9	0,513	0,780	0,342
SL-HAN-TS	123	7,7	0,537	0,812	0,339
SL-JUR-TS	110	6,9	0,561	0,761	0,263
SL-JUG	120	7,6	0,582	0,804	0,276
SL-OLYM-TS	50	3,1	0,439	0,843	0,480
SL-RYB-TS	84	5,3	0,560	0,741	0,244
SL-BREN-TS	90	5,6	0,583	0,781	0,253
SL-VOJN-TS	50	3,1	0,613	0,812	0,245
Mean	108,02	6,75	0,525	0,759	0,308

If in a population the frequency of genes and genotypes are permanent generation by generation, it is in Hardy-Weinberg equilibrium. Each examined population differs in each locus from the Hardy-Weinberg equilibrium (HWE). We determined the deviations on three significancy levels (P<0,05; P<0,01; P<0,001). Each examined locus differs from HWE in the TR-KIV-TRA population. In the case of MAF35, MAF65, MAF70, TGLA53, TGLA357, BM1314, OarCP20 and OarFCB20 locus are on P<0,001 level; MCMA7, INRA127, OarCP49 and BM6506 the level of locus are on P<0,01; and the MCM527, ILSTS11 and OarAE119 is on P<0,05. We determined deviation from HWE in SL-OLYM-TS population on the CSSM 43, TGLA53 and MCM527 locus, while in the SL-VOJN-TS population on MAF 70 locus.

On the examined locus we determined 50 population specific alleles in 21 populations. Preservation of these alleles require special attention, because they are typify only definite population. These alleles were not determined on CSSM43 and MAF65 locus.

The most informative markers are TGLA357 and OarCP49, because in those 8 and 7 population specific alleles were determined. Population specific alleles were determined in the following population (with the number of specific alleles in the parenthesis): HU-KMKK-AC (2,) HU-PG-ZC (2), HU-LB-TZC (1), HU-SZIC-AC (1), HU-SMA-AC (5), HU-DE-CSC (3), HU-OJ-TC (1), BU-STAR-TS (4), BU-ROD-TS (1), BU-PFMAR (1), BU-WFMAR (5), BU-PLBH (3), RO-RUST-TS (2), RO-RUDA (1), AL-TS (1), AL-RUDA (1), AL-BARDH (2), SL-HAN-TS (1), SL-VAN-TS (1), SL-JUR-TS (3), SL-RYB-TS (1), SL-SIR-TS (1), TR-KIV-TRA (3), SM-CS-TS (2), SM-KRI-PR (1), SM-SVR-PR (1).

We constracted genetic distance matrix - on the grounds of Nei standard genetic distance (D_S) and minimum genetic distance values (D_M) – in order to identify the genetic connection among examined population. This short thesis is not contain the matrix because of its size.
The largest distance was found between Serbian Zomborski (SM-ZP-TS) and two Slovakian and one Turkish Sakiz population (SL-VOJN-TS-0.356, TR-SAKIZ-0.315, SL-RYB-TS-0.306).
Among Hungarian population the farest genetic distance were found: HU-LB-TCZ and HU-KMKK-AC (0.175), and HU-KMNP-AC (0.194); HU-PG-TRC and HU-KMKK-AC (0.148), and HU-KMNP-AC (0.166). The genetic distance was unimportant between the two population belonging to Kőrös-Maros National Park (HU-KMKK-AC, HU-KMNP-AC) (0.028) and HU-SMA-AC, HU-OJ-TC (0.034). The population from Makó-Rákos (HU-MRD-TAC) is regarded as the transition of milking and autochthonous type. According to our results it has negligible genetic difference from the autochthonous HU-SZIC-AC population (0.045) and has nearly the same difference from all the other examined population.
The examined Bulgarian populations are close to each other. The nearest genetic relation was found between the two Tsigai (BU-STAR-TS and BU-ROD-TS) (0.053) and two Maritza (BU-PFMAR and BU-WFMAR) (0.056). The Pleveny Blackhead sheep (BU-PLBH) has closest genetic relation to Patch Faced Maritza sheep (BU-PFMAR) (0.0618) and has the farest relation to Rodopski Tsigai (BU-ROD-TS) (0.087).
The Slovakian SL-VOJN-TS population differs from all other examined Slovakian populations. In our opinion the result can not be explained with the low number of

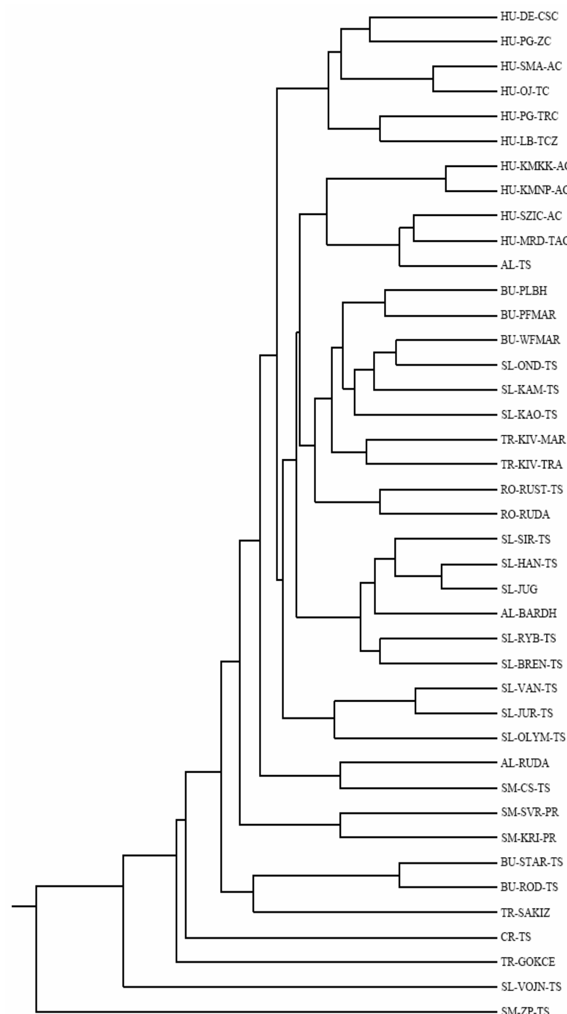
examined individuals (5), because we did not get unexpected results, when we examined other populations with the similar low number. Among the 12 examined Slovakian populations the genetic differene was negligible: SL-JUG and SL-HAN-TS (0.03) as well as SL-JUG and SL-JUR-TS (0.042).

The examined Albanian, Turkish and Serbian populations genetically differ from each other. The examined two Rumanian populations (RO-RUDA, RO-RUST-TS) are genetically similar (0.065), the distance between Albanian and Rumanian Ruda populations (RO-RUDA and AL-RUDA) is not much more, but not negligible (0.087). Among the two Turkish Kivircik populations (TR-KIV-MAR, TR-KIV-TRA), - which are from two regions - and the Serbian Pramenka populations (SM-SVR-PR, SM-KRI-PR) are moderately differentiated (0.072 and 0.086). The Croatian Tsigai (CR-TS) and Serbian Zomborski (SM-ZP-TS) population are highly differentiated from the 39 examined populations.

These genetic relationships are reflected on the phylogenetic tree, constructed from genetic distance (D_M) data using the UPGMA algorithm (Figure 1). Tree can demonstrate the previously written informations. The Serbian Zomborski and the Croatian Tsigai differ from all the examined populations. The two Bulgarian Tsigai (BU-STAR-TS and BU-ROD-TS) are in very close relation and they next to the Turkish Sakiz (TR-SAKIZ), which belongs to the Zackel type. The Kivircik populations (TR-KIV-TRA and TR-KIV-MAR from two Turkish regions) are close to each other and on the phylogenetic tree they belong to the same branch with the Bulgarian Pleveny Blackhead sheep (BU-PLBH), two Bulgarian Maritza (Patch Faced Maritza (BU-PFMAR), White Faced Maritza (BU-WFMAR) and three Slovakian Tsigai (SL-KAO-TS, SL-KAM and SL-OND-TS). Among Rumanian populations, the Rumanian Ruda (RO-RUDA) has the closest relation to Rumanian Rusty Tsigai (from Turda-RO-RUST-TS). The Hungarian examined Tsigai populations are on two branches of tree. On one of them two smaller branches can be distinguish, where the two autothchtonous populations (HU-KMKK-AC and HU-KMNP-AC) –from Kőrös-Maros National Park-, and HU-SZIC-AC population and the population from Makó-Rákos (HU-MRD-TAC) are found. The Albanian Tsigai (AL-TS) is teh closest relation with them. The other branch,which contains the Hungarian flocks, also devides into two sub-branches containing two-two populations. Population from Soltszentimre (HU-SMA-AC) and Akasztó (HU-OJ-TC) are close relationship although the previous as a autothchtonous one, the latter as a milking one according to the official register. The Rusty Tsigai (HU-

PG-TRC), which was crossed with Merino; has the closest relation to the Milking population from Cegléd (HU-LB-TCZ). Population from Csóka (HU-DE-CSC) is closeset to an other Milking population (HU-PG-ZC). In Hungary the population from Cegléd is regarded as the most typical Milking type, which were developed with Serbian (Zomborski and Cokanski) bucks in the last 15 years. Among Slovakian population on one branch of the tree there are the SL-JUR-TS and SL-VAN-TS, which are genetically near to the SL-OLYM-TS. On the other branch of the tree there are the SL-JUG, SL-HAN-TS and SL-RYB-TS, SL-BREN-TS populations. The SL-SIR-TS is in the nearest connection with SL-JUG and SL-HAN-TS. The Albanian Bardhoke sheep (AL-BARDH) is near to these, which belongs to the Zackel type. Far from these population there is an other Slovakian group. SL-KAO-TS, SL-KAM-TS and SL-OND-TS belong to this group. The SL-VOJN-TS differs not only from the other examined Slovakian, but from all examined population.

Figure 1: UPGMA phylogenetic tree of the examined populations



The results of study of SLA-6, -7 and -8 genes

The results of gene expression work

Preparation of study

Before the result of real gene expression research we show the beginning of the work. Quantitative real-time PCR method was used, which needs appropriate quality and quantity of cDNA and gene specific primers. After the total RNA isolation we checked their quality (1% agarose gel, RNA 6000 Nanochip-Bioanalyzer Agilent), and their quantity (NanoDrop).

In the case of MHC Ib genes we constructed the possible primer pairs by Primer Express, Primer 3 program and manually. It was not so easy , because the homology of the sequences was high. During the manual designing we aimed beside the standard criteria of selection primers, the 3' tails of the primers gene specific attribute too. The criteria in case of SLA-1 gene were similar but here we planned the forward primer into the exon 5 and the reverse primer into the junction (exon 5 and 6). In case of SLA Ib genes the forward and reverse primers were design into exon 2, junction and exon 3.

After the test of their gene specific attribute, one primer pair per gene were chose.

Absolute quantification was used to determine the efficiency of the PCR reaction, to create standard curves and to find the best cDNA concentration.

During relative quantification it was important to select the best housekeeping genes for normalize the data. It was a hard task, because the expression of these genes is the same in all tissue and cell types and in all experimental condition, physiologycal state. After testing 5 genes (Beta – actin (ACTB); Beta – 2 microglobulin (B2M); 18S rRNA; Glyceraldehide – 3 – phosphate dehidrogenase (GAPDH); Hipoxantin guanin phosphoribosil transferase (HPRT1)) the most optimal was the ACTB and B2M, but in reality we used B2M during the normalization of our data in order to save money and time.

The results of real gene expression study

The classical SLA-1 and the three non classical MHC I genes (SLA-6, SLA-7, SLA-8) were expressed in each examined tissue. The expression of SLA-1 gene was multiple more in each tissue than the expression of non classical genes. The difference of the expression level was hundred, thousand fold in several case. The expression of SLA-1

gene was the least level in the thymus in all case. Figure 2., 3., 4. show the expression of non classical genes.

Figure 2: Expression of SLA-6, SLA-7 and SLA-8 genes in different tissues from adult sow

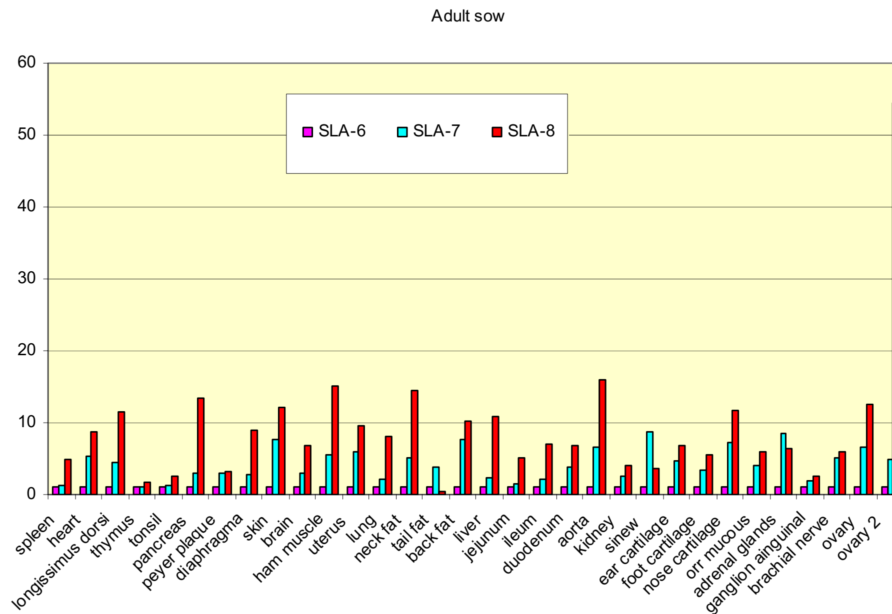


Figure 3: Expression of SLA-6, SLA-7 and SLA-8 genes in different tissues from adult boar

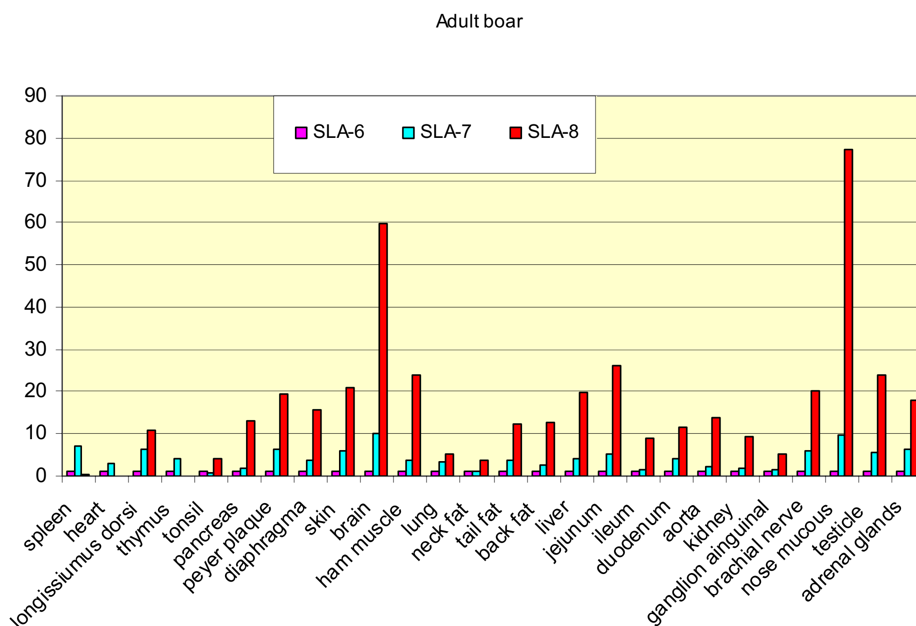
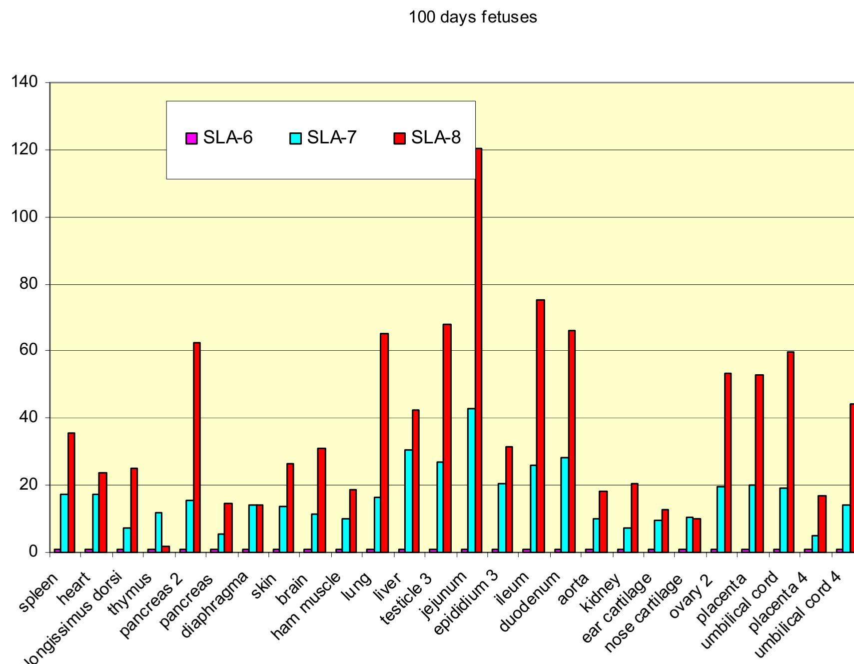


Figure 4: Expression of SLA-6, SLA-7 and SLA-8 genes in different tissues from 100 days fetuses



As the above figures show the non classical MHC I genes were expressed in each examined tissue. It was in contrast to results of CREW et al (2004). According them SLA-6 expressed in all 11 samples, which were examined, and SLA-8 expressed in both except brain and SLA-7 had tissue specific expression. Almost in all examined tissue SLA-8 had the highest expression level, the second was the SLA-7 and finally the SLA-6, but it was also expressed. In thymus SLA-7 expressed in the highest level in boar and fetus. In sow the expression of SLA-7 was stronger in case of other tissues (sinew, tail fat, adrenal gland). In fetus the previously tissues were not examined, in boar tail fat and adrenal gland were examined, and the expression of SLA-8 was stronger. Expression of SLA-7 was stronger in spleen and heart in boar.

All non classical MHC I genes were expressed in all tissues on mRNA level using quantitative real time PCR method.

The results of polymorphism study

Preparation of the study

Polimorphism study was started with genespecific primer design and its confirmation. Primer 3 program was used for design primers. Our aim was the amplify the whole exon 2, because it is the most polimorphic region in human, and we supposed it is true in the case of pig.

The real results of polymorphism study

In case of SLA-6 gene one non conservative mutation was detected inside exon 2, at 529 position. There is a citosine, adenine substitution, which causes proline glutamine aminoacid substitution. It causes a modification of molecule folding. We detected this mutation in the following Chinese breeds: Guizou, Lantang, Min, Yimeng, Wuzhi Shan, Xiang, Dawei.

In case of SLA-7 one new allele was detected, but this is inside intron 1. There is a timin citosine substitution at position 371.

Inside exon 2 of SLA-8 gene, at position 383 one citosine adenine substitution was identified. This conservative mutation does not cause aminoacid substitution. It was detected in Chinese Laiwu, Meshian and European Large White pig.

As the result of our polimorphism study we can confirm, that SLA-6,-7,-8 genes are belong to the group of non classical genes, as we have identified only one new allele per a gene, which means really low level polimorphism and this is the main character of non classical genes. But we have to emphasise, that we examined only one individual per breed, so we can not state yet, that these new alleles are specific for the breed. We are planning to continue this study to examine more breeds and individuals.

V. NEW SCIENTIFIC RESULTS

In the first part of the thesis genetic difference among the Tsigai and Zackel sheep populations were estimated using 16 microsatellite markers. In the second part polimorphism of non classical MHC I genes on DNA level and its expression on mRNA level using quantitative real-time PCR method in different European and Chinese pig breeds were studied.

The statements of genetic distance estimate are the following:

1. We stated that 16 microsatellite markers from 20, which are on different chromosome, easily and quickly can determine the genetic difference among Tsigai and Zackel sheep populations.

2. We confirmed the genetic difference –which is based on previous results of phenotype and blood biochemistic polymorphism research- among Hungarian Tsigai populations.

3. We stated, that the genetic difference is *unimportant* among:
   - the two flock (HU-KMKK-AC, HU-KMNP-AC) owned by Kőrös-Maros National Park;
   - population from Soltszentimre (HU-SZIC-AC) –registered as autochthonous- and population from Akasztó (HU-OJ-TC) –registered as a Milking;
   - population from Csanádpalota (HU-SZIC-AC) –registered as autochthonous- and population from Makó-Rákos (HU-MRD-TAC);
   - the Slovakian Handel (SL-HAN-TS) and Jugat (SL-JUG-TS); the Jurbis (SL-JUR-TS) and Jugat (SL-JUG-TS); the Vancover (SL-VAN-TS) and Jurbis (SL-JUR-TS) populations.

4. We stated that the genetic difference is *very low (but not negligible)* among:
   - the two Serbian Pramenka populations (SM-SVR-PR, SM-KRI-PR);
   - the two Kivircik populations (TR-KIV-TRA, TR-KIV-MAR) from different Turkish region;
   - the two Bulgarian Tsigai populations (BU-STAR-TS, BU-ROD-TS);

- the two Bulgarian Maritza populations (BU-PFMAR, BU-WFMAR);
- the Rumanian and Albanian Ruda (RO-RUDA, AL-RUDA);
- the Albanian Tsigai (AL-TS) and Hungarian population from Csanádpalota (HU-SZIC-AC) and from Makó-rákos (HU-MRD-TAC).

5. We stated that the genetic difference is *on high level*:
    - among the Serbian Zomborski population (SM-ZP-TS) and all the other examined population. We confirmed the results of Cinkulov (2003), who studied the genetic difference between Serbian Cokanski and Zomborski, and determined that the genetic difference between these two population is so extended, that these can be regarded as two seperate breed.

6. According to our results we can not confirm the definite opinion of Draganescu (2003), that the Tsigai breed is from Romanian Ruda. However we pointed out, that some examined Tsigai populations have genetic relation with it (RO-RUST-TS, TR-KIV-TRA, SL-HAN-TS, BU-WFMAR, BU-PFMAR).

Conclusions of SLA Ib genes expression and polimorphism study:

- Results of the gene expression study

1. We stated that the primers – designed by us – are genespecific and the housekeeping genes – selected by us - are suited for quantitative RT PCR research.

2. We stated that the SLA-6,-7,-8 genes were expressed in all examined adult and fetal tissue on mRNA level.

3. We stated that the expression of classical SLA-1 gene has the highest expression level in all examined tissue, in several cases more than hundred or thousand times more than SLA Ib genes.

4. We stated that among non classical genes the expression of SLA-8 gene is the highest, after the SLA-7 and SLA-6 has the lowest expression level. The only except is the thymus, because in this case the expression of SLA-7 gene was the highest.

- Results of polimorphism study

1. We stated that primers which were designed by us, are genespecific and adapted for our work.

2. In case of all SLA Ib gene we identified one new allele:
   SLA-6 1 non conservative mutation inside exon 2 (c529a), P99Q
   SLA-7 1 mutation inside intron 1 (c371t)
   SLA-8 1 conservative mutation inside exon 2 (c383a)

3. We found that the SLA-6, -7, -8 genes have very low polimorphism.

4. We confirmed its not classical gene classification.

VI. PRACTICAL UTILITY OF THE RESULTS

1. In order to preserve the various Tsigai and Zackel populations, we have to know its genetic structure, differences. Further gene loosing can be avoided using our results from genetic difference study of sheep population. And genetic variablity can be growing. In knowledge of inbreeding value of examined population the farmers can directly use them in practice.

2. We do not have too much informations about non classical MHC I genes in neither species. With our results we contribute to have more information about SLA Ib genes. The knowledge of expression of the SLA Ib genes in distinct tissues is important for the study of the difficulty of xenotransplantation. The molecular trait of polymorphism specifies whether the immunreaction against antigenes comes into action or not. Mutations which were detemined in polymorphism study can have effect on the sequence, structure and function of protein too.

VII. PUBLICATION LIST

<u>*Reviewed scientific publications*</u>

- **Sz. Kusza**- Zs. Bősze- S. Kukovics- A. Jávor (2003): Molecular genetic assays of CAE infection in Hungarian goat flock. Prospects for the 3rd Millenium Agriculture. Cluj Napoca. 9-11 October.2003.
- S. Kukovics- A. Molnár- M. Ábrahám- Z. Dani- **Sz. Kusza**- Gy. Fülöp- A. Jávor (2003): Presence of CAEV infection in Hungarian goat industry as an effect of livestock import. Prospects for the 3rd Millenium Agriculture. Cluj Napoca. 9-11 October.2003.
- **Kusza Sz**.- Bősze Zs.- Kukovics S.- Jávor A. (2004): A CAE-ra való fogékonyság vizsgálata. Acta Agraria Debreceniensis. 33-36 pp.
- **Sz. Kusza**- Zs. Bősze- S. Kukovics- A. Jávor (2004): Genetic Assay of CAE in the Hungarian Goat Herd. South African Journal of Animal Science. 34.(Supplement 1) 15-18 pp. (IF: 0,38)
- Gy. Veress- **Sz. Kusza**- Zs. Bősze- S. Kukovics- A. Jávor (2004): Polymorphism of the αs1-casein, k-casein and β-lactoglobulin gene in the Hungarian Goat Herd. South African Journal of Animal Science. 34.(Supplement 1) 22-25 pp. (IF: 0,38)
- S. Kukovics- A. Molnár- M. Ábrahám- Z. Dani- **Sz. Kusza**- Gy. Fülöp- Zs. Bősze- A. Jávor (2004): Effects of CAEV infection on the performance of Hungarian goat breeds. South African Journal of Animal Science. 34.(Supplement 1) 227-229 pp. (IF: 0,38)
- **Kusza Szilvia**- Veress Gyula- Kukovics Sándor- Jávor András- Sanchez Armand- Angiolillo Antonella- Bősze Zsuzsanna (2006): Genetic polymorphism of αs1- and αs2-caseins in Hungarian Milking Goats. Small Ruminant Res. (in press) (IF: 0,777)
- **Sz. Kusza**- I. Nagy- Zs. Sasvári- A. Stágel- T. Németh- A. Molnár- K. Kume- Zs. Bősze- A. Jávor- S. Kukovics (2006): Genetic diversity and population structure of Tsigai and Zackel type of sheep breeds in the Central-, Eastern- and Southern-European regions. Small Ruminant Research (under acceptance)
- **Sz. Kusza**- P. Chardon- Zs. Bősze- A.Jávor- C. Rogel-Gaillard (2006): Study of non-classical MHC class I genes in pigs. First European Conference on Pig Genomics. Lodi. Olaszország. 2006. február 20-21.

- Veress Gy.- **Kusza Sz.**- Kukovics S.- Jávor A.- Bősze Zs. (2006): A kecske kazein gének polimorfizmusa. Kérődző állatfajok mai helyzete és perspektívái az Európai Unióban. SZIE, Gödöllő. 2006. 04.10.-11.

Conference proceedings in Hungarian

- Kukovics S.- Molnár A.- Ábrahám M.- Dani Z.- **Kusza Sz**.- Fülöp Gy. (2003): A magyarországi kecskefajták CAE érintettsége. EU konform mezőgazdaság és élelmiszerbiztonság. Gödöllő, 219-228 pp.

Conference proceedings in foreign language

- S. Kukovics- A. Molnár- M. Ábrahám- Z. Dani- **Sz. Kusza**- Gy. Fülöp (2003): Presence of CAEV infection in Hungarian goat industry as an effect of livestock import. EAAP-54th Annual Meeting. Rome. 31. Aug-3. Sept. 2003. 320 pp.

Other publications

- Kukovics S.- Molnár A.- Ábrahám M.- Dani Z.- Fülöp Gy.- **Kusza Sz**.- Bősze Zs.- Jávor A. (2003): A hazai kecskeállomány CAE érintettségének meghatározása, valamint géntechnológiára (DNS vizsgálatok) alapozott betegség mentesítési- és megelőzési rendszerének kidolgozása. Kutatási jelentés. OM.
- **Kusza Sz.**- Jávor A. (2003): A cigája fajtafenntartásának lehetőségei és indokai. Az állattenyésztés szolgálatában. Debrecen. 189-194 pp.
- **Kusza Sz**.- Jávor A. (2004): A kecske AIDS-e. Magyar Juhászat+Kecsketenyésztés. A Magyar Mezőgazdaság melléklete. 2004. 13. évf. 7. szám. 4-6 pp.
- **Kusza Sz.**- Kukovics S.- Jávor A. (2006): A cigája változatok közötti genetikai távolság becslése. Génmegőrzés (szerk.: Mihók S.). DE-ATC. 64-70 pp.