

## SNPS OF CALPAIN/CALPASTATIN SYSTEM GENES IN COMMERCIAL POPULATION OF ABERDEEN ANGUS IN KHARKIV REGION, EASTERN UKRAINE

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**Summary.** The calpain proteolytic system — the micromolar calcium-activated neutral protease  $\mu$ -calpain encoded by the CAPN1 gene, and its inhibitor — calpastatin — encoded by the CAST gene, is involved in biological processes regulation by partial degradation of substrates — cytoskeletal proteins, signaling molecules and enzymes. Calpain system dysfunction caused by mutations or  $\text{Ca}^{2+}$  elevation is associated primarily with diseases affecting central nervous and cardiovascular systems, as well as skeletal muscles. For cattle breeding purposes the calpain hyperactivity in the presence of excess calcium ions (calcium passes out due to cell death) is considered to be desirable offering the possibility to obtain more tender meat. This study is aimed to analyze the polymorphic variants CAPN316 and CAST282 of calpain and calpastatin genes in Aberdeen-Angus bred within Kharkiv region and to compare obtained result with commercial herds of other countries.

For the SNP genotyping, PCR-RFLP methods were set up. Testing deviation from the Hardy-Weinberg equilibrium was performed using Pearson's chi-squared test. Spearman's correlation coefficient was used to measure the strength of association between two characteristics. Cluster analysis was used to classify the allele frequencies obtained within similar data obtained for cattle in other countries.

The allele and genotype frequencies of SNP CAPN316 (AF252504.2:g.5709C>G) in calpain gene are: C — 0.398 and G — 0.602; CC — 13.6%, CG — 52.3% and GG — 34.1%. The allele and genotype frequencies of SNP CAST282 (AY\_008267.1:g.282C>G) in calpastatin gene are: C — 0.807 and G — 0.193; CC — 63.6%, CG — 34.1% and GG — 2.3%. Group-to-group variability in allele C frequency for SNP CAPN316 (0.37%) is higher than CAST282 (0.06%), due to the modulating effect of the calpastatin. The Aberdeen-Angus herd of Kharkiv region are comparable to the European commercial beef cattle herds by allele frequencies. Association between latitudinal zonation and frequency of allele C in cattle herds was demonstrated ( $R = 0.53$ ,  $p < 0.05$ ).

**Keywords:** Aberdeen-Angus breed, calpain gene, calpastatin gene, SNP, meat tenderness

**Introduction.** Mammalian calpain protease family includes more than 15 types of enzymes, being universal calcium-dependent, non-lysosomal cysteine proteases, acting as modulators of cell molecules function by selective degradation of proteins (Smith and Schnellmann, 2012). Calpains are ubiquitously present in cells and tissues of vertebrates as different isoforms (Goll et al., 2003). Calpain substrates are enzymes, signaling molecules and cytoskeletal proteins. Calpain activation occurs at neutral pH and depends on free calcium level. Different calpains require micromolar ( $\mu$ -calpain) or millimolar (m-calpain)  $\text{Ca}^{2+}$  concentration (Grebinyk,

2012). Calpastatin is a specific inhibitor of calpain, activated by  $\text{Ca}^{2+}$  concentration required to achieve the half of  $\mu$ -calpain maximal activity. Skeletal muscle calpain/ calpastatin system includes three proteases — calpain I ( $\mu$ ), calpain II (m), and calpain 3 (p94), and their inhibitor calpastatin. The large catalytic subunit of calpain is encoded by three genes CAPN1, CAPN2, CAPN3 and small regulatory subunit is encoded by CAPN4 gene (Nowak, 2011).

Calpains are involved in biological processes regulation such as cellular differentiation, apoptosis, synaptic transmission, muscle protein metabolism etc.

(Sato and Kawashima, 2001), therefore calpain activity dysfunction mediated by calcium metabolism disorders or by mutations in calpain or calpastatin genes will be accompanied by pathological condition (Sakamoto et al., 2013). Increased calpains levels have been detected in various pathophysiological processes such as ischemia-induced tissue damage of heart (e.g., myocardial infarction), kidney, lung, liver or central nervous system (e.g., stroke), inflammations, muscular dystrophies, cataracts, diabetes, Alzheimer's disease, Huntington's disease, Parkinson's disease, multiple sclerosis (Goll et al., 2003), and tumors of the urinary system and bladder in cattle (Roperto et al., 2010), infectious diseases such as malaria and diseases caused by protozoa (Samanta et al., 2012; Li et al., 2007).

Investigating calpain system in animals as a model objects allows to simulate the physiological processes at the cellular and organismal level providing the high proteolytic activity of calpain in humans, additionally, function of calpain system has an economic value in cattle breeding. An important aspect of the calpain and calpastatin localized in the cattle muscle tissue is impact on meat quality expressed as tenderness appearing during postmortem proteolysis. After cell death  $Ca^{2+}$  concentration gradually increases and reaches the sufficient level for calpain activation, initiating the destruction of the muscle fibers in the lateral and transverse sections. Animals selection based on meat tenderness without molecular genetic studies is difficult, because this feature is not possible to estimate during animal's life. In this paper we analyzed *CAPN1* gene as the enzyme encoded by this gene is activated by less  $Ca^{2+}$  concentration than needed for calpain II activation. Calpain II is predominantly localized in the cytosol, but 70% of calpain I is bound with myofibrils — that ensures more effective scaffold proteins destruction during postmortem proteolysis. Since the calpain I is suppressed by calpastatin *CAST* gene was included in analysis too.

There are several SNP of *CAPN1* and *CAST* studied in cattle worldwide up to date: *CAPN316* (AF252504.2:g.5709C>G), *CAPN530* (AF\_288054.2:g.4558G>A), *CAPN4751* (AF\_288054.2:g.6545C>T), *CAST2959* (AF\_159246.1:g.2959A>G), *CAST2870* (AF\_159246.1:g.2870A>G), *CAST282* (AY\_008267.1:g.282C>G) (Gill et al., 2009; Ribeca et al., 2009). However, the studies of these genes in cattle within neighboring countries are of inadequate attention. In Ukraine, SNP *CAPN530* was studied for six beef breeds (Dobrianska et al., 2013), in Russia - SNP *CAPN316* for Kalmyk cattle (Kosyan et al., 2012). In Czech Republic, SNP *CAPN1* c.947G> C and *CAST* c.155C>T were studied for seven beef breeds (Kaplanová et al., 2013). The search and selection of candidate loci for *CAPN1*

and *CAST* genes is carried out in Poland (Juszczuk-Kubiak et al., 2004) and in other countries (Barendse et al., 2007). Papers concerning the calpastatin gene in Ukrainian cattle or cattle of CIS countries were not found.

The aim of this study was to analyze the allele frequencies of genotypes for a polymorphic variant gene *CAPN316* *CAPN1* and *CAST282* *CAST* gene in a group of animals Aberdeen-Angus.

This study is aimed to analyze the polymorphic variants *CAPN316* and *CAST282* of calpain and calpastatin genes in Aberdeen-Angus bred within Kharkiv region and to compare obtained result with commercial herds of other countries.

**Material and methods.** The study object were Aberdeen-Angus cattle (n = 88; cows: n = 82, bulls: n = 6) bred at PE 'Agrofirma Svitank', Kharkiv region, Ukraine. DNA was extracted from blood samples using DNA extraction kits 'Diatom DNA Prep 100' ('Isogene', Russia). For the SNP genotyping, PCR-RFLP methods were set up, using primer pairs (Miquel et al., 2009; Schenkel et al., 2006). Restriction enzymes used were endonuclease *RsaI* and *BtgI* ('Fermentas', Lithuania). The digested fragments were electrophoresed on 2.0% agarose gel.

The deviation of allele frequencies from Hardy-Weinberg equilibrium was tested using Pearson's chi-squared test. For data distribution it was used normality test assessment. Spearman's correlation coefficient was used to measure the strength of association between two characteristics. The distance between two clusters in dendrogram was defined as an average distance between all pairs of objects therein, using sizes of the respective clusters as a weigh coefficients and Euclidean metric as a distance function (Khalafyan, 2007).

**Results.** Allele frequencies of SNPs *CAPN316* and *CAST282* for Aberdeen-Angus herd studied is given in Table 1.

**Table 1** - Allele frequencies at two SNPs in the Aberdeen-Angus herd of Kharkiv region

Aberdeen-Angus	Allele frequencies			
	<i>CAPN316</i>		<i>CAST282</i>	
	C	G	C	G
Cows (n = 82)	0.390	0.610	0.793	0.207
Bulls (n = 6)	0.500	0.500	1.000	0.000
Total (n = 88)	0.398	0.602	0.807	0.193

C to G alleles ratio of SNP *CAPN316* for bulls and for whole sampling population were not significantly differ from the 1:1 ratio. C allele frequency of SNP *CAST282*

was quadruple to that of *G* allele for the whole sample and for cows. There were no bulls with allele *G*. *C* alleles of both SNP are associated with a loose muscle tissue structure following incubation at 5 °C, and respectively, with higher meat tenderness (Schenkel et al., 2006, Leveau, 2008).

The cytidine/guanosine (*C/G*) polymorphism in exon 9 of the gene *CAPN1*, which results in the amino acid substitution of glycine with alanine in large catalytic subunit of  $\mu$ -calpain. Consequently, the enzyme activity in muscle tissue increases, resulting to the decrease of myofiber stiffness due to postmortem proteolysis. Expression of *C* allele in *CAST* gene, intron 5, position 282 results to non-functional calpastatin molecule synthesis. Therefore, when the  $Ca^{2+}$  concentration becomes sufficient to activate calpain inhibition of calpain does

not occur and myofiber proteolysis prolongs. *C* alleles of both SNPs are associated with beef tenderness (Schenkel et al., 2006). Thus, the SNPs alleles distribution in population studied suggests moderate catalytic activity of calpain I and weak calpastatin inhibitory activity. The meat obtained from these animals will become more tender during prolonged storage period, because calpain activity is not inhibited by calpastatin non-functional molecules.

When tested deviation from the Hardy-Weinberg equilibrium using Pearson's chi-squared test there were no statistically significant differences between actual and expected genotype frequencies for both genes (see Table 2). The genotype frequencies in group studied was found to be at equilibrium.

**Table 2** – Genotype frequencies at two SNPs in the Aberdeen-Angus herd of Kharkiv region

Object	Parameter	CAPN1			CAST		
		CC	CG	GG	CC	CG	GG
Cows (n = 82)	n <sub>act.</sub>	10	44	28	50	30	2
	%	12.2	53.7	34.1	61.0	36.6	2.4
	n <sub>exp.</sub>	12.5	39.0	30.5	57.3	27.4	3.3
	%	15.2	47.6	37.2	62.9	32.8	4.3
	Statistics	$\chi^2_{act.} = 1.346; p > 0.05$			$\chi^2_{act.} = 1.049; p > 0.05$		
Bulls (n = 6)	n <sub>act.</sub>	2	2	2	6	0	0
	%	33.3	33.3	33.3	100.0	0	0
	n <sub>exp.</sub>	1.5	3.0	1.5	6.0	0	0
	%	25.0	50.0	25.0	100.0	0	0
	Statistics	$\chi^2_{act.} = 0.667; p > 0.05$			-		
Total (n = 88)	n <sub>act.</sub>	12	46	30	56	30	2
	%	13.6	52.3	34.1	63.6	34.1	2.3
	n <sub>exp.</sub>	14.0	42.0	32.0	57.3	27.4	3.3
	%	15.9	47.9	36.2	65.1	31.2	3.7
	Statistics	$\chi^2_{act.} = 0.715; p > 0.05$			$\chi^2_{act.} = 0.788; p > 0.05$		

Note: df=2,  $\Sigma\chi^2_{st.} = 5.99$ .

The analysis of SNPs *CAPN316* and *CAST282* within Aberdeen-Angus group studied and literature data for Aberdeen-Angus and other breeds was performed (Tables 3–4).

According to the literature sources (Table 3), the maximal allele frequency *C* for *CAPN1* gene was observed in the Canadian Aberdeen-Angus population

(0.71), and the minimal — in the Argentinian population (0.09). The highest allele frequency for SNP *CAST282* is observed in Ukrainian Aberdeen-Angus group (Table 4). Generally, the mentioned groups are characterized by relatively high frequency of *C* allele. Low frequency of *C* allele for *CAPN1* gene (less than 0.10) in Argentinian and Brazilian groups can be explained by several reasons.

**Table 3** – Allele frequencies at *CAPN316* in the Aberdeen-Angus worldwide and other breeds by literature sources

Country	Breed	n	CAPN316		Reference
			C	G	
Canada	Aberdeen-Angus	158	0.71	0.29	Carruthers, 2009
International population <sup>1</sup>	Aberdeen-Angus	26	0.63	0.37	Carruthers, 2009
USA	Aberdeen-Angus	213	0.41	0.59	Van Eenennaam et al., 2007
Ukraine	Aberdeen-Angus	88	0.40	0.60	Present study
Sweden	Aberdeen-Angus	13	0.31	0.69	Leveau, 2008
Scotland	Aberdeen-Angus (50%) <sup>2</sup>	430	0.22	0.78	Gill et al., 2009
Argentina	Aberdeen-Angus	11	0.09	0.91	Soria et al., 2010
Spain	Retinta	89	0.64	0.36	Avilés et al., 2013
	Charolais	98	0.36	0.64	
	Limousin	99	0.32	0.68	
USA	Beef cattle <sup>3</sup>	1209	0.17	0.83	Quaas et al., 2006
Brazil	Nellore	638	0.08	0.92	Pinto et al., 2006

Notes: <sup>1</sup> International population represents an elite selection of international Angus cattle, the embryos donated by top purebred producers from Australia, Brazil, Denmark, Ireland, Scotland, Uruguay and the USA were implanted to Canadian cows. <sup>2</sup> Aberdeen Angus-sired beef cattle. <sup>3</sup> Hereford-sired, Charolais-sired Angus cattle

**Table 4** – Allele frequencies at *CAST282* in the Aberdeen-Angus worldwide and other breeds by literature sources

Country	Breed	n	CAST282		Reference
			C	G	
Ukraine	Aberdeen-Angus	46	0.81	0.13	Present study
Sweden	Aberdeen-Angus	8	0.75	0.25	Leveau, 2008
Scotland	Aberdeen-Angus (50%) <sup>1</sup>	442	0.64	0.36	Gill et al., 2009
Canada	Aberdeen-Angus	12	0.63	0.37	Schenkel et al., 2006
USA	Beef cattle <sup>2</sup>	1209	0.72	0.28	Quaas et al., 2006
Spain	Charolais	98	0.76	0.24	Avilés et al., 2013
	Retinta	89	0.67	0.33	
	Limousin	99	0.65	0.35	
Spain	Piemontese	109	0.61	0.39	Ribeca et al., 2009
Brazil	Nellore	638	0.63	0.37	Pinto et al., 2006
Brazil	Nellore	290	0.55	0.45	Gomes et al., 2013
Turkey	Turkish Grey Steppe	132	0.51	0.49	Kök et al., 2013

Notes: <sup>1</sup> Aberdeen Angus-sired beef cattle. <sup>2</sup> Hereford-sired, Charolais-sired Angus cattle

The climate conditions of South America suggest that according Bergman's rule mammals inhabiting warmer climate seem to be smaller-bodied, than animals inhabiting cold climates. *C* allele for *CAPN1* gene is associated with hyperactive calpain synthesis involved in the formation of gaps in the microfibrils during the growth of muscle tissue, thus animals with *CC* and *CG* genotypes will have advanced muscle mass. Calpastatin is associated with pleiotropic effect on carcass composition (Bolormaa et al., 2016).

Moreover, high calpain activity is associated with protozoa diseases, such as malaria and Visceral Leishmaniasis. In turn, the calpain inhibitors slow down the development of protozoa diseases. These diseases are prevalent in tropical and sub-tropical zones, therefore cattle would rather have alleles of normally active calpain and calpastatin in these region. For both SNPs studied these are *G* alleles. Additionally, calpain inhibitor has negative effect on IL-1 $\alpha$  release, depressing immune

function (Goll et al., 2003). This would justify smaller dispersion observed for *C* allele frequency of SNP *CAST282*, than *CAPN316*. Smaller dispersion can be explained by calpastatin modulating function, therefore *C* allele frequency distribution of SNP *CAST282* will have a more conservative pattern.

The traditional 'breeder's approach' can also explain resulting allele frequencies, because selection of phenotypic traits did not increase of *C* allele frequency in the South American cattle sample populations.

Aberdeen Angus, originated from Scotland are adapted to sufficiently cold conditions, therefore, this breed is likely to have high *C* allele frequency of SNP *CAPN316* which did not fix in a population inhabiting a warmer climate zone. Ukrainian Aberdeen-Angus by *C* allele frequency is similar to the North American population. Table 5 shows the *C* allele frequencies for SNPs *CAPN316* and *CAST282* considered as advantageous in terms of meat tenderness.

**Table 5** – *C* allele frequencies at *CAPN316* and *CAST282* in the Aberdeen-Angus worldwide and other breeds by climate zones (Longitudinal length)

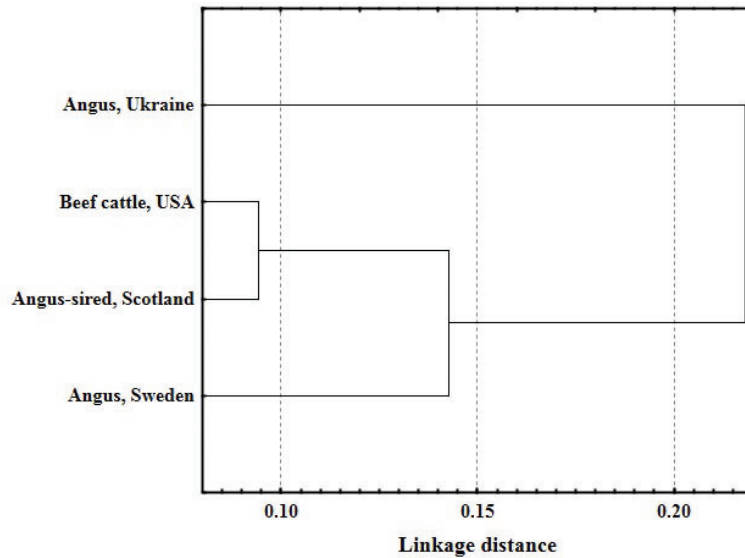
Country	Longitudinal length	C allele, SNP CAPN316	C allele, SNP CAST 282
Sweden	63°N	0.31	0.75
Scotland	57°N	0.22	0.64
Canada	56°N	0.71	0.63
Ukraine	49°N	0.40	0.81
USA	40°N	0.17; 0.41	0.72
Spain	40°N	0.32; 0.64; 0.22	0.76; 0.65; 0.67; 0.61
Turkey	39°N	—	0.51
Argentina	37°S	0.09	—
Brazil	11°S	0.08	0.63; 0.55
Spearman's correlation coefficient			
	R	0.39	0.53 *
	t	1.34	1.97 *

Note: \* -  $p < 0.05$ ;  $df=10$ ,  $t_{st} = 1.82$ .

*C* allele frequency of SNP *CAST282* is associated with latitudinal zonation, i. e. allele frequency is rather determined by a combination of climatic factors than animal breed. This trend is supported by previously described role of calpain inhibitor for immune response and susceptibility to the protozoan diseases.

Cluster analysis included only data on cattle groups having been studied for both *CAPN316* and *CAST282*. Since most papers describe different calpain and calpastatin genes SNPs composition for wide variety

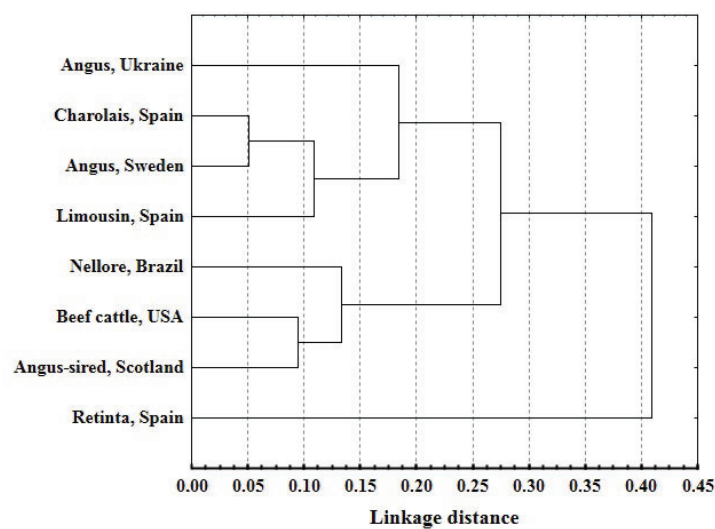
of cattle breeds there were found three papers for Aberdeen-Angus herds simultaneously studied for SNPs *CAPN316* and *CAST282*. Dendrogram (Fig. 1) showed Eastern Ukrainian herd to be separated from other groups. Dendrogram shape indicates that given groups do not form distinct classes; the closest groups are of hybrid cattle having 50% genes of Aberdeen-Angus breed or less. Purebred Swedish and Ukrainian Aberdeen-Angus herds are less similar to each other than crossbred animals.



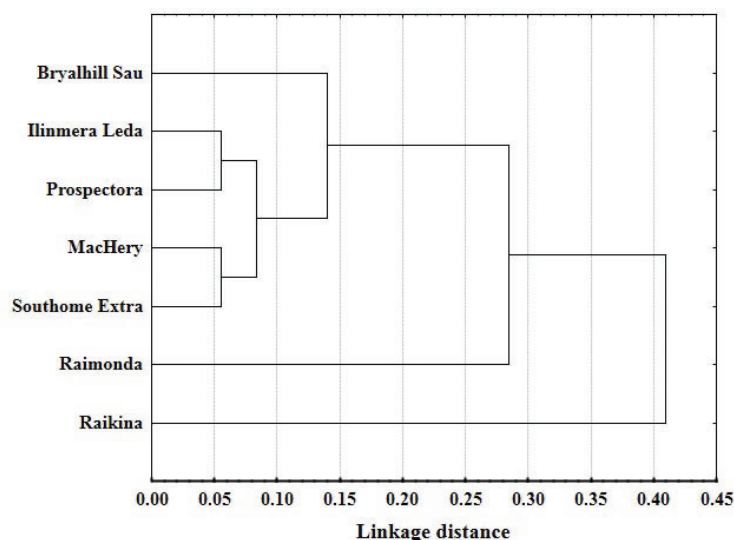
**Figure 1.** Dendrogram of Aberdeen-Angus based on *CAPN316* and *CAST282* (based on literature data and present study)

Extended analysis was based on papers describing SNPs *CAPN316* и *CAST282* for other beef breeds (Fig. 2). There are two distinct groups on elongated diagram ‘continental’ group (European countries) and ‘island’ (non-European countries). Aberdeen-Angus herds of different countries were not allocated in one class, groups formation is rather based on geographic region then on breed. Eastern Ukrainian Aberdeen-Angus herd is close to the ‘European’ class, but there is a significantly greater distance between Ukrainian group than intra-group distance within class. The minor distance between groups within ‘European’ class refers to

a greater similarity between them. Crossbred Aberdeen-Angus groups (50% or less) are more likely to be similar to Brazilian Nellore. The results obtained suggest that microevolutionary processes are tend to select the advantageous allele profile for specified climate and geographical conditions rather than ancestry history. Thus SNPs allele distribution of European Anguses is closer to that ones of other domestic European breeds than to Scottish sample population. Ukrainian sample population seems to be isolated from European herds due to later introduction and more continental conditions of Eastern Ukraine.



**Figure 2.** Dendrogram of beef cattle based on *CAPN316* and *CAST282* (based on literature data and present study)



**Figure 3.** Dendrogram of Aberdeen-Angus lines of Eastern Ukrainian herd on *CAPN316* and *CAST282*

The Aberdeen-Angus group studied is produced under Canadian selection and predominately includes animals belonging to lines: Bryalhill Sau, Ilinmera Leda, MacHery, Prosectora, Raikina, Raimonda, Southome Extra (Kolisnyk et al., 2014). The highest C allele frequency of SNP *CAPN316* was reported for Raikina line - 0.67 compared with other lines — from 0.35 to 0.45 (see Table 6). The highest C allele frequencies of SNP *CAST282* were reported for Raikina and Southome Extra lines — 0.83. The lines do not form distinct classes (Fig. 3).

**Conclusion.** The preferred C allele frequencies of SNPs *CAPN316* and *CAST282* in the study population were 0.398 and 0.807, the population is in state of equilibrium. The genotype frequencies of *CAPN316* are CC — 13.6%, CG — 52.3% and GG — 34.1%, of *CAST282* CC — 63.6%, CG — 34.1% and GG — 2.3%. The population studied is close to European commercial beef herds. Group-to-group variability in allele C frequency for SNP *CAPN316* is higher than *CAST282*, due to the modulating effect of the calpastatin. Calpain/calpastatin system genes are likely to be associated with meat tenderness as well as some quantitative traits selected during beef cattle breeding. It was demonstrated the role of climate and geographic region on C/G distribution

of SNPs rather than breed belonging, and, moreover, association between latitudinal zonation and frequency of allele C in cattle herds ( $R = 0.53$ ,  $p < 0.05$ ).

**Table 5** – Allele frequencies at two SNPs in the Aberdeen-Angus herd of Kharkiv region, by lines

Aberdeen-Angus line	CAPN316		CAST282	
	C	G	C	G
Bryalhill Sau (n = 10)	0.35	0.65	0.90	0.10
Ilinmera Leda (n = 13)	0.38	0.62	0.73	0.27
MacHery (n = 9)	0.44	0.56	0.78	0.22
Prosectora (n = 21)	0.38	0.62	0.81	0.19
Raikina (n = 3)	0.67	0.33	0.83	0.17
Raimonda (n = 3)	0.17	0.83	0.67	0.33
Southome Extra (n = 9)	0.44	0.56	0.83	0.17

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