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PHYSICAL LOCATION OF GENES ENCODING SMALL HEAT SHOCK PROTEINS IN THE *SUIDAE* GENOMES*

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The subject of the studies carried out was physical mapping of the HSPB1, HSPB2, CRYAB (alternative name HSPB5), HSPB6 and HSPB8 genes from the family of small heat shock protein genes (HSPB) on chromosomes of the domestic pig (Sus scrofa domestica) and European wild pig (Sus scrofa scrofa). The application of FISH technique with probes derived from porcine BAC clones: CH242-237N5, CH242-333E2, CH242-173G9 and CH242-102C8 made it possible to determine the location of the studied genes, respectively, in 3p15, 9p21, 6q12 and 14q21 genome regions of domestic and wild pigs. The physical localization of HSPB genes allowed assigning these loci to the linkage and syntenic groups of genes in Suidae. Precise, molecular and cytogenetic identification of genes responsible for resistance to stress and disease, and determining meat production is essential for the genetic selection effects, aimed to reduce mortality causing significant economic loss in animal production. The studies performed may help to elucidate the role of the HSPB genes in protection against pathogenic or environmental stress, affecting pigs' survivability and meat quality.

Key words: Suidae, FISH, HSPB genes, muscle development, meat quality

Small heat shock proteins (HSPB) are the smallest, most variable in size, class of the multigene heat shock protein (HSP) family, having molecular masses ranging approximately from 15 to 30 kDa and the α-crystallin domains (~85 amino acids residues) in the highly conserved C-terminal protein regions. HSPB (1–10) stress-associated proteins, constitutively present in most cells, exert chaperone-like activity under normal physiological conditions as well as protective functions against

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cellular stress, in particular that which alters protein folding (Taylor and Benjamin, 2005). Amongst them, the HSPB1, CRYAB (HSPB5), HSPB6 and HSPB8 are ubiquitously expressed in different neuronal and non-neuronal tissues, whereas expression of HSPB2 is essentially restricted to heart and muscle (Mymrikov et al., 2011). Each tissue has a unique transcription profile of these stress proteins, regulated during development and differentiation, and resulting from specific functions such as modulation of the cytoskeleton and inhibition of apoptosis (Wettstein et al., 2012; Arrigo, 2013). Therefore, the impaired HSPB expression, caused by functional mutations of the encoding genes, has pathological consequences involving neuropathies, myopathies and immunosuppressive disorders (Arrigo, 2012; Boncoraglio et al., 2012; Benndorf et al., 2014; Dubińska-Magiera et al., 2014).

Recently, expression of six members of the small heat shock protein family (HSPB1, HSPB2, CRYAB, HSPB6, HSPB7 and HSPB8) has been analysed in the nervous and non-nervous tissues (lens, brain, heart, liver, kidney, lung, skeletal muscle, stomach, colon) of the pigs at several stages of ontogeny (from full-term fetuses to three-year-old adult), which were used as models to study the impact of different forms of stress (hypoxia, bacterial infection, physical activity, transport) on postnatal expression of these proteins (Tallot et al., 2003; Verschuure et al., 2003; Chiral et al., 2004; Golenhofen et al., 2004; Nefti et al., 2005; David et al., 2006; Bao et al., 2008, 2009; Jensen et al., 2012; Liu et al., 2014). The experiments revealed that impairment of *HSPB* genes expression affects stress response and results in severe adverse developmental outcome, neonatal morbidity and mortality as well as death syndrome of transported slaughter pigs and poor eating quality of meat. The latest studies proved chaperone and anti-apoptotic role of HSPB proteins during conversion of pig muscle to meat which is believed to ultimately influence meat quality, with a special consideration of tenderness (Lametsch and Bendixen, 2001; Hwang et al., 2005; Herrera-Mendez et al., 2006; Ouali et al., 2006; Kwasiborski et al., 2008; Laville et al., 2009; Lomiwes et al., 2014).

Chromosomal localization of the *HSPB* genes is a good tool to identify additional, new QTLs associated with pig stress and disease resistance, feed efficiency, product quality and reproductive performance. Furthermore, it may be also a basis for developing genetically modified strains with improved production traits or providing transgenic model animals for human diseases and therapy (Whyte and Prather, 2011; Hu et al., 2013).

Assembled genome sequences are available for *Suidae* species, however due to existence of many gaps or errors in gene locations, it is important to verify these assemblies (e.g. Sscrofa10 build representing 98% of porcine genome) using fluorescence *in situ* hybridization (FISH) technique. This method of cytogenetic mapping makes it possible to improve physical maps as well as enhance the quality and applicability of whole genome sequences for genetic analysis (Lewin et al., 2009; Jiang et al., 2013).

The aim of the presented study was chromosomal assignment of the *HSPB1*, *HSPB2*, *CRYAB* (*HSPB5*), *HSPB6* and *HSPB8* small heat shock protein genes in the genomes of domestic and wild pigs.

Material and methods

Cytogenetic preparation and chromosome identification:

Blood samples were collected from 9 healthy domestic pigs (*Sus scrofa domestica*) of 990 hybrid line and 4 European wild pigs (*Sus scrofa scrofa*) (after culling within the framework of the planned wildlife management – Act of 13 November 1995 on Hunting Laws, Journal of Laws of 1995 No. 147, item 713, as amended; Journal of Laws of 2015, item 2168, of 2016 item 1082). Lymphocytes were cultured and treated for late BrdU and H33258 incorporation to obtain DAPI-banded chromosome preparations for FISH detection (Iannuzzi and Di Berardino, 2008). Chromosome identification followed the standard karyotype, according to the international nomenclature for the domestic pig chromosomes (Gustavsson, 1988).

Probe preparation and FISH:

The porcine Bacterial Artificial Chromosome (BAC) clones, overlapping five small heat shock protein genes: *HSPB1*, *HSPB2*, *CRYAB* (*HSPB5*), *HSPB6* and *HSPB8*, were obtained from the CHORI-242 Porcine BAC Library (http://www.chori.org/bacpac/porcine242.htm). The presence of the studied genes in clones, selected based on information about BAC end sequences (BES) (http://www.sanger.ac.uk/Project-s/S_scrofa/BES.shtml), was verified by PCR using gene-specific primers (Table 1).

Table 1. BAC clones used in FISH experiments and PCR protocols verifying presence of the studied *HSPB* genes

Gene	Clone name	GenBank accession	PCR			
			Primers	Ta, °C	Prod. size, bp	Gene fragm.
HSPB1	CH242-237N5	AY789513	F: 5’ ctc gaa aat aca cgc tgc cc 3’ R: 5’ gga tgg tga tct ctg ccg ac 3’	57	129	exon 3
HSPB2	CH242-333E2	DN119723	F: 5’ ttg ccc tca cta agc cga ag 3’ R: 5’ ggc cac cac tga gta cga g 3’	58	186	exon 3
CRYAB	CH242-333E2	DY408556	F: 5' cca ttc aca gtg agg acc cc 3’ R: 5’ ccg cct ctt tga cca gtt ct 3’	59	378	exon 1–2
HSPB6	CH242-173G9	AY574050	F: 5’ ttt ctc ggt gct gct gga tg 3’ R: 5’ gca tgc acc tcc aca tgt tc 3’	59	84	exon 1
HSPB8	CH242-102C8	AY609863	F: 5’ ctc tct gag cct ccg ttt cc 3’ R: 5’ tgc tgc ttc tcc tcg tgt tt 3’	56	429	exon 1

It was not possible to select separate clones for the closely located *HSPB2* and *CRYAB* (*HSPB5*) genes in the pig genome, therefore the same clone (CH242-333E2) containing sequences of both genes was used (http://www.ncbi.nlm.nih.gov/gene).

The BAC DNA was isolated, labelled with biotin 16-dUTP by random priming and used as probes in the FISH experiments on chromosomes of *Suidae* species. Labelled probes with an excess of porcine competitor DNA were denatured for 10 min at 70°C, preannealed for 30 min at 37°C, and applied onto chromosome preparations. Hybridizations were carried out overnight at 37°C.

Signal detection and amplification were performed using avidin-FITC anti-avidin system. Slides were stained by DAPI and analysed under fluorescence microscope (Nikon) equipped with computer-assisted image analysis system (Cyto Vision).

Results

Strong, positive FITC signals were obtained after fluorescence *in situ* hybridization with all BAC clones used as probes, with frequency of FITC signals (double or single spots on both or single chromosomes or chromatids) varying from 81% (*CRYAB*) in the domestic pigs to 32% (*HSPB2*) in the wild pigs.

FISH-mapping facilitated the successful assignment of five *HSPB* genes to the following porcine chromosome regions: SSC3p15 (*HSPB1*), SSC9p21 (*HSPB2* and *CRYAB*), SSC6q12 (*HSPB6*) and SSC14q21 (*HSPB8*) (Figure 1). The studied *loci* were identified on different chromosomes, extending the cytogenetic maps for chromosome 3, 9, 6 and 14 of the studied *Suidae* species.

The *HSPB2* and *CRYAB* (*HSPB5*) genes (clustered at the distance of 0.863 kb) were both mapped to the identical SSC9p21 pig genome region, just as human homologues of these genes (located only about 0.9 kb apart), which were assigned in the corresponding HSA11q22-q23 chromosome band (Iwaki et al., 1997). Close, head-to--head linkage of the *HSPB2*/*CRYAB* gene pair, raising a possibility of shared regulatory elements for their expression, is a conserved feature of the mammalian genomes (Doerwald et al., 2004).

Discussion

The five small heat shock protein genes studied were mapped earlier by the linkage mapping approach to a specific pig chromosome, but band-specific location was not determined (Humphray et al., 2007; Jiang and Rothschild, 2007; Vingborg et al., 2009). The physical assignments of the *HSPB* genes presented in this study correspond with these general findings and are in agreement with cytogenetic localization in the human genome, if human (HSA) – pig (SSC) comparative chromosome painting data are considered (https://www-lgc.toulouse.inra.fr/pig/compare/HSA.htm) (Goureau et al., 1996) (Table 2). Furthermore, the results obtained are consistent with our previous provisional comparative mapping of these genes in the genomes of domestic and wild pig species (Danielak-Czech et al., 2014).

On the whole, the experiments reported in this paper definitely proved that FISH--based mapping is still useful to validate the data on physical gene location, construct precise genome maps and improve pig genome assemblies (Lewin et al., 2009; Jiang et al., 2014) (http://www.ncbi.nlm.nih.gov/projects/genome/guide/pig/).

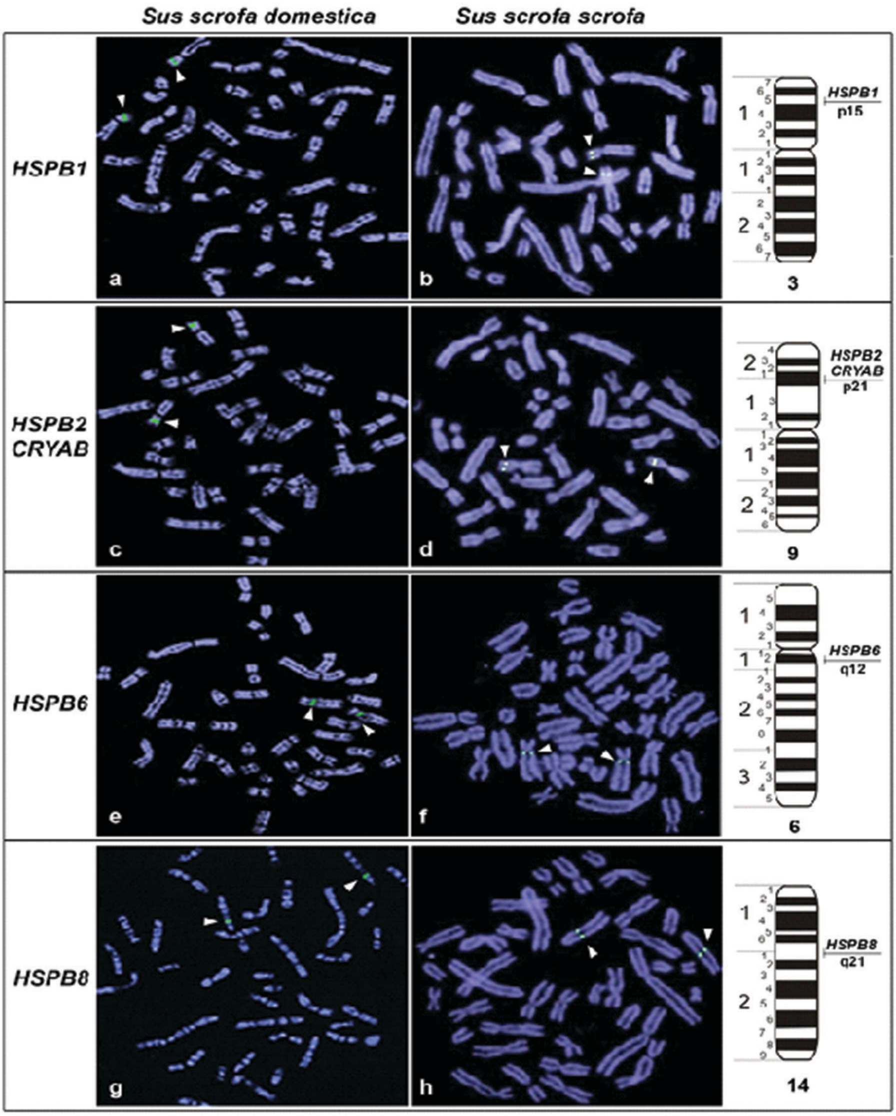

Figure 1. Cytogenetic localization of *HSPB* genes (shown by arrows) on the domestic and wild pig chromosomes: *HSPB1* (a, b), *HSPB2* and *CRYAB* (c, d), *HSPB6* (e, f), HSPB8 (g, h)

This study adds further information not only to the previous genetic, physical or integrated pig genome maps but also to the QTL maps (http://www.animalgenome.org/QTLdb/pig.htlm) and precisely assigns five loci encoding small heat shock proteins on chromosomes of the domestic and wild pigs, which are the major *Suidae* species of economic importance (Hu et al., 2013; Rothschild et al., 2007; Hu et al., 2009).

During the last few years, the significant changes of the *HSPB* gene expression were reported to be involved in tissue-specific (brain, heart, skeletal muscle and gastrointestinal tract) stress response in developing piglets and adult slaughter pigs (David et al., 2000; Tallot et al., 2003; Verschuure et al., 2003).

Table 2. Cytogenetic location of the studied *HSPB* genes in the domestic and wild pigs as well as human genomes and functions of encoded proteins

Gene symbol	Gene name	Protein function	Cytogenetic location	
			SSC	HSA
HSPB1	heat shock 27kDa protein 1	stress resistance, actin organization	3p15	7q11.23
HSPB2	heat shock 27kDa protein 2	somatic muscle development, stress response	9p21	11q22–23
CRYAB	crystallin, alpha B	anti-apoptosis, response to heat, muscle development, negative regulation of intracellular transport, camera-type eye development, structural constituent of eye lens, unfolded protein binding and homooligomerization	9p21	11q22.3–23.1
HSPB6	heat shock protein beta-6	stress response, structural constituent of eye lens, protein homodimerization	6q12	19q13.12
HSPB8	heat shock 22kDa protein 8	chaperone activity, stress response, identical protein binding	14q21	12q24.23

The experiments, carried out on a piglet model of perinatal hypoxia, showed markedly increased level of *HSPB1* and *HSPB6* gene transcripts in brain (cerebellum, cortex and hippocampus) as well as overexpression of *CRYAB* (*HSPB5*) gene in heart (left ventricle) and gastrointestinal tract (stomach, colon) at piglet birth (David et al., 2000; Chiral et al., 2004; Nefti et al., 2005; Louapre et al, 2005). Besides, the newest studies performed on a swine-specific *in vitro* infection model revealed high *HSPB1* gene expression in intestinal porcine epithelial cells of newborn and weaning piglets, induced by probiotics which counteract the pathogenic effects of enterotoxigenic bacteria (Liu et al., 2014, 2015). The elevated expression of these genes was proved to protect neonatal and post-weaning pigs against hypoxia and intestinal disorders, which are the crucial morbidity and mortality reasons of the perinatal developing and young pigs. On the other hand, expression of *HSPB1* and *CRYAB* genes in adult slaughter pigs, submitted to stressful events like transport, was found to decline and cause increased susceptibility to acute heart failure and the sudden death syndrome in transported pigs (Bao et al., 2008, 2009). In this context, the identification of porcine stress protein genes, controlling stress and disease resistance, is important in view of the fact that pigs are good model animals for studying human diseases, involving therapy and prevention.

The important aspect of nowadays research becomes the relationship between HSPB protein expression and meat quality. A substantial body of evidence suggests that transportation or pre-slaughter physical stress, related to low expression of the *HSPB1* and *CRYAB* genes in skeletal muscles, result in deterioration in meat quality associated with higher temperature, lower pH and increased drip loss, which subsequently lead to reduced colour and water-holding capacity (not affecting pork toughness) (Jensen et al., 2012; Young et al., 2009; Yu et al., 2009; Tang et al., 2014). Many latest studies demonstrate and notably emphasize the emerging role of *HSPB* proteins during the conversion of muscle to meat, as the factors that regulate the process of apoptotic cell death of muscle cells and ultimately influence eating quality of meat (Herrera-Mendez et al., 2006; Ouali et al., 2006; Lomiwes et al., 2014). Concretely, it is suggested that due to chaperone function in maintaining protein integrity, the down regulation of *HSPB1* and *CRYAB* genes influences the proteolytic degradation of actin and myosin which result in increasing meat tenderness, juiciness and flavour, whereas the higher concentrations of these proteins favour darker meat colour and cooking loss (Hwang et al., 2005; Kwasiborski et al., 2008; Lomiwes et al., 2014; Bernard et al., 2007).

It is worth to note that four of the cytogenetically mapped *HSPB* genes reported in this paper (except *HSPB2*), were localized within or near many QTLs for meat and carcass quality traits (http://www.animalgenome.org/cgi-bin/QTLdb/SS/index) involving: flavour, colour, odour, pH, stiffening and texture (meat water holding capacity and tenderness, carcass temperature). A majority of these QTLs (over thirty) were found in the vicinity of the *HSPB6* (SSC6) and *HSPB8* (SSC14) genes, while only several (nine) close to the *HSPB1* (SSC3) *locus*. The chromosomal region covering the SSC9p21 band, where *CRYAB locus* was mapped, is rather poor in QTLs, therefore only 4 for meat flavour and texture were identified (just as in the case of *UCP2* and *UCP3* genes mapped in our earlier studies) (Kozubska-Sobocińska et al., 2014). Due to the biological function of encoded proteins and their location overlapping QTL regions for the pig meat quality traits, the studied *HSPB* genes can be considered as candidates for such traits.

In conclusion, the physical localization of *HSPB* genes in the *Suidae* genomes is of great importance for improving the physical maps and enhancing the quality of whole genome sequence assemblies, contributing their applicability for genetic analysis. Moreover, the genomic location data of *HSPB* genes may be a basis for studies on their polymorphism underlying product quality traits, with particular emphasis on eating quality of pig meat. In addition, the identification of porcine *loci* controlling susceptibility to specific stress and diseases, including cytogenetic mapping of *HSPB* genes, opens possibilities to develop genetically modified pig models for studying human perinatal dysfunctions, cognitive impairments, developmental delays and carcinogenesis.

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Fizyczna lokalizacja genów kodujących małe białka szoku cieplnego w genomach *Suidae*

STRESZCZENIE

Przedmiotem przeprowadzonych badań było fizyczne mapowanie genów *HSPB1*, *HSPB2*, *CRYAB* (alternatywna nazwa *HSPB5*), *HSPB6* oraz *HSPB8* z rodziny małych białek szoku cieplnego (*HSPB*) na chromosomach świni domowej (*Sus scrofa domestica*) i dzika europejskiego (*Sus scrofa scrofa*). Zastosowanie techniki FISH z sondami uzyskanymi ze świńskich klonów BAC (CH242-237N5, CH242-333E2, CH242-173G9, CH242-102C8) umożliwiło określenie lokalizacji badanych genów, odpowiednio, w regionach 3p15, 9p21, 6q12 i 14q21 genomów świni domowej i dzika. Fizyczna lokalizacja genów *HSPB* pozwoliła na przyporządkowanie tych loci do sprzężeniowych i syntenicznych grup genów u *Suidae*. Precyzyjna, molekularna i cytogenetyczna identyfikacja genów odpowiedzialnych za odporność na stres i choroby oraz warunkujących produkcję mięsa jest istotna dla selekcji genetycznej, mającej na celu obniżenie śmiertelności powodującej znaczne straty ekonomiczne w produkcji zwierzęcej. Przeprowadzone badania mogą przyczynić się do wyjaśnienia roli genów *HSPB* w ochronie przed patogennym lub środowiskowym stresem, wpływającym na przeżywalność świń i jakość mięsa.

Słowa kluczowe: *Suidae*, FISH, geny *HSPB*, rozwój mięśni, jakość mięsa