

GENE POLYMORPHISM FREQUENCIES IN KRŠKOPOLJE PIG BREED

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Abstract: Genetic polymorphisms are associated with different production traits in pigs and some of them have major effects. In local pig breeds, which are usually characterised by small effective sizes, elimination of unfavourable alleles is more challenging because care must be taken to maintain genetic diversity. Therefore, the aim of the present study was to analyse the frequency of several causal mutations in the local breed Krškopolje pig associated with fatness and meat quality and to estimate the selected diversity parameters. A total of 253 DNA samples of Krškopolje pigs were analysed with Choice Genetics' custom SNP-chip. We observed a relatively high frequency (0.17) of stress syndrome mutation (mutated c. 1843T allele at *RYRI*). The mutated c.749A allele at *PRKAG3* gene (Arg250Gln or RN) associated with high muscle glycogen deposition was absent. The frequency of the variant at *PRKAG3* (Ile249Val) encoding 249Ile (associated with higher pH, fatness, darker colour, better water holding capacity) was 0.22, and of the variant encoding 102Ser was 0.50. The frequency of *MC4R* 892A allele associated with high lipid deposition and growth was 0.57. The average observed and expected heterozygosity values were 0.37 and 0.36, respectively, which is relatively high for a local pig breed. The present results demonstrate high frequencies of both unfavourable and favourable alleles affecting meat quality in Krškopolje pigs. Further monitoring of key polymorphisms and genetic diversity parameters is needed to develop conservation and breeding strategies.

Key words: local pig breed, candidate genes, SNPs, breeding program

Introduction

In recent decades, the main focus in pig breeding has been on improving carcass leanness, growth and reproductive performance, but this process was carried out only on a limited number of breeds (*Tribout et al., 2010; Rauw et al.,*

1998). As a result, many local pig breeds experienced population decline or even extinction. Nowadays, local pig breeds are regaining importance due to increased societal awareness of the need to preserve phenotypic and genotypic diversity, better adaptive capacity to specific local environmental conditions and the potential for production of high-quality traditional meat products (Čandek-Potokar and Nieto 2019).

The Slovenian local Krškopolje pig is an example of such a breed, typically raised on small farms with only a few breeding sows (Batorek Lukač *et al.*, 2019). As it has not been subjected to genetic selection, the breed exhibits higher carcass fatness, low growth potential and muscle development (Poklukar *et al.*, 2023). An increased interest of farmers for selective breeding of local breeds (including Krškopolje pig) has been noted in a recent survey (Mercat *et al.*, 2022) mainly in terms of reproductive performance and maintenance of genetic diversity, with meat quality also mentioned. However, breeding for meat quality in Krškopolje pig represents a great challenge due to the small population size and demanding organization of post-mortem sampling and measurements. Therefore, identification and characterization of molecular markers affecting these traits of interest is necessary for the development of breeding procedures aimed at managing genetic information. The main aim of the present study was to analyse the frequency of several causal mutations known to affect fatness and meat quality traits in the local Krškopolje pig, and to estimate the selected genetic diversity parameters which will help to develop conservation and breeding strategies.

Materials and Methods

Samples of ear tissue of 253 Krškopolje pigs were collected at slaughter for DNA extraction. Animals originated from 26 sires, 93 dams and 15 breeding farms (from 1 to 59 animals per farm). Genomic DNA was extracted using the Qiamp DNA Mini kit (Qiagen GmbH, Hilden, Germany) following manufacturer instructions. The DNA samples were genotyped using Choice Genetics' custom SNP-array containing 57811 single nucleotide polymorphisms (SNPs). For all samples, the average genotyping rate was more than 0.98. Genotype quality control and data filtering was performed using PLINK 1.9 software (Purcel *et al.*, 2007). SNPs with minor allele frequencies less than 0.01 or with more than 10% of missing genotypes were excluded from the analysis. After filtering, 51304 SNPs remained, out of which 49651 SNPs were located on *Sus scrofa* 11.1 autosomes.

Allele frequencies and genetic diversity parameters, such as observed and expected heterozygosity, inbreeding coefficient of an individual relative to the subpopulation (F_{is}) and minor allele frequency with all SNPs were calculated. Principal component analysis (PCA) of genotyping data according to the breeding

farm was performed using the R environment (version 4.3.1). Six causative SNPs associated with meat quality and fatness (Table 1) were extracted from the genotyping data and their allele frequencies were individually studied.

Table 1. Studied polymorphisms and their documented association with production traits

Gene	Chr	Genome position (<i>Sus scrofa</i> 11.1)	Polymorphism	Trait	Reference
<i>RYR1</i>	6	47357966	c.1843C>T	Meat quality	<i>Fujii et al. 1991</i>
<i>PRKAG3</i> (Arg250Gln or RN')	15	120863533	c.749G>A	Meat quality	<i>Milan et al., 2000;</i> <i>Ciobanu et al. 2001</i>
<i>PRKAG3</i> (Ile249Val*)	15	120863537	c.745G>A	Meat quality	<i>Milan et al., 2000;</i> <i>Ciobanu et al. 2001</i>
<i>PRKAG3</i> (Pro103Leu**)	15	120864863	c.158C>T	Meat quality	<i>Milan et al., 2000;</i> <i>Ciobanu et al. 2001</i>
<i>PRKAG3</i> (Gly102Ser***)	15	120865227	c.304G>A	Meat quality	<i>Milan et al., 2000;</i> <i>Ciobanu et al. 2001</i>
<i>MC4R</i>	1	160773437	c.892G>A	Backfat and growth	<i>Kim et al., 2000</i>

Chr = chromosome, * previously Ile199Val, ** previously Pro53Leu, *** previously Gly52Ser

Results and Discussion

Allele frequencies of six polymorphisms related to meat quality and carcass traits were studied in Krškopolje pig (Table 2). As previously shown (*Muñoz et al., 2018; Tomažin et al., 2021*), we confirmed a relatively high frequency (i.e. 0.17) of mutation 1843C>T in the ryanodine receptor 1 (*RYR1*) gene (*Fujii et al., 1991*), of which the negative effects on technological quality of pork are well known (*Salmi et al., 2010*). The mutated T allele is associated with uncontrolled loss of calcium from the sarcoplasmic reticulum to the cytosol, affecting the rate of pH decline and consequently meat water-holding capacity and colour (*Fujii et al., 1991*). The presence of the *RYR1* mutated allele has been reported to be very low or absent in some European local pig breeds and in some cases, effective initiatives to eliminate this allele were taken (*Muñoz et al., 2018*).

As for protein kinase AMP-activated non-catalytic subunit gamma 3 (*PRKAG3*) gene, four polymorphisms that affect meat quality have been studied in

Krškopolje pigs (Table 2). In its active form, the PRKAG3 enzyme inhibits glycogen synthesis and stimulates glycogen breakdown (Milan *et al.*, 2000; Ciobanu *et al.*, 2001). The presence of the dominant PRKAG3 c.749A mutated allele (Arg250Gln or RN) has been associated with high muscle glycogen levels resulting in low ultimate pH, poor water-holding capacity and pale meat colour (Milan *et al.*, 2000). The mutated c.749A allele was absent in Krškopolje pig (Table 2) which is consistent with previous work (Muñoz *et al.*, 2018). The PRKAG3 c.745G>A mutation (or Ile249Val) was associated with muscle ultimate pH, colour, fatness and water-holding capacity (Ciobanu *et al.*, 2001, Enfält *et al.*, 2006). In Krškopolje pig, it was segregating with the frequency of favourable A allele of 0.22, thus we confirm the previously reported frequency of this allele on much smaller sample of Krškopolje pig (Muñoz *et al.*, 2018). In PRKAG3 gene, there were two additional mutations reported that cause amino acid substitutions, i.e. Leu103Pro (c.158C>T) and Gly102Ser (c.304G>A) (Milan *et al.*, 2000), and have a potential effect on meat quality. In Krškopolje pig, C allele of PRKAG3 c.158C>T substitution was fixed, while the frequency of PRKAG3 c.G304A (Gly102Ser) was 0.5 (Table 2).

Table 2. Allele frequencies for six polymorphisms on candidate genes for meat quality, growth and fatness in Krškopolje pig breed

	Allele frequency (N = 253)
<i>RYRI</i>	
C allele	0.83
T allele	0.17
<i>PRKAG3</i> (Arg250Gln)	
G allele	1.00
A allele	0.00
<i>PRKAG3</i> (Ile249Val)	
G allele	0.78
A allele	0.22
<i>PRKAG3</i> (Leu103Pro)	
C allele	1.0
T allele	0.0
<i>PRKAG3</i> (Gly102Ser)	
G allele	0.50
A allele	0.50
<i>MC4R</i>	
G allele	0.43
A allele	0.57

The melanocortin-4 receptor (*MC4R*) is involved in the regulation of feed intake and energy balance. A missense mutation c.892G>A in *MC4R* gene replaces aspartic acid with asparagine and causes dysfunction of the protein. Its mutation (i.e. A allele) was previously associated with increased fatness, feed intake and with growth differences (Kim *et al.*, 2000; Piórkowska *et al.*, 2010). In the present study, the frequency of the A allele was intermediate (i.e. 0.57), which corroborates with the previous study involving Krškopolje pig (Muñoz *et al.*, 2018).

The average minor allele frequency of all genotyped SNPs (Figure 1) in Krškopolje pig was 0.274, with 11669 highly informative SNP markers with frequencies between 0.4 and 0.5 and 7030 SNP markers with frequencies between 0.01 and 0.10.

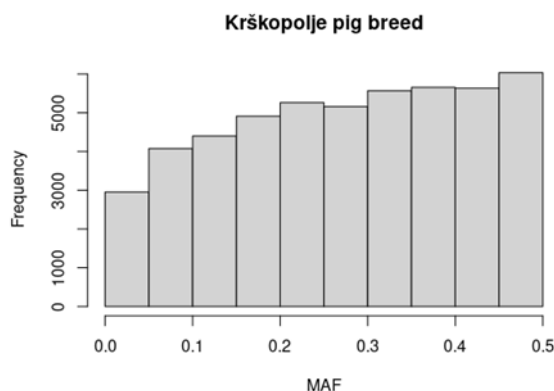


Figure 1. Frequency distribution of minor allele frequencies (MAF) in Krškopolje pig population

Genetic variability parameters (Table 3) within Krškopolje pig demonstrated relatively high expected and observed heterozygosity for local pig breeds, i.e. 0.360 and 0.370, respectively. Similar values for the Krškopolje pig were also reported by Muñoz *et al.* (2019) using different SNP-array and a smaller number of animals in the analysis. The observed and expected heterozygosity levels were higher than in other European local and similar to modern pig breeds (Herrero-Medrano *et al.*, 2014; Muñoz *et al.*, 2019; Bordonaro *et al.*, 2023). In the present study, the F_{is} value was observed to be negative (i.e. -0.03), indicating that there are fewer homozygotes than expected by random mating.

Table 3. Genetic diversity parameters of Krškopolje pig

Krškopolje pig (n = 253)	Mean
Observed heterozygosity	0.370
Expected heterozygosity	0.360
F_{is}	-0.030

F_{is} = inbreeding coefficient of an individual relative to the subpopulation

PCA was employed to explore the clustering of individuals from different breeding farms (Figure 2). The first and second principal components explained 10.44 and 8.38 % of the total variation, respectively. PCA separated two breeders of Krškopolje pigs (i.e. Breeder-A and Breeder-O) from the others. This could be a result of different breeding strategies or isolation of farm populations from the other farms. However, larger number of genotyped animals from different breeding farms is needed to see if or why these Krškopolje subpopulations are isolated.

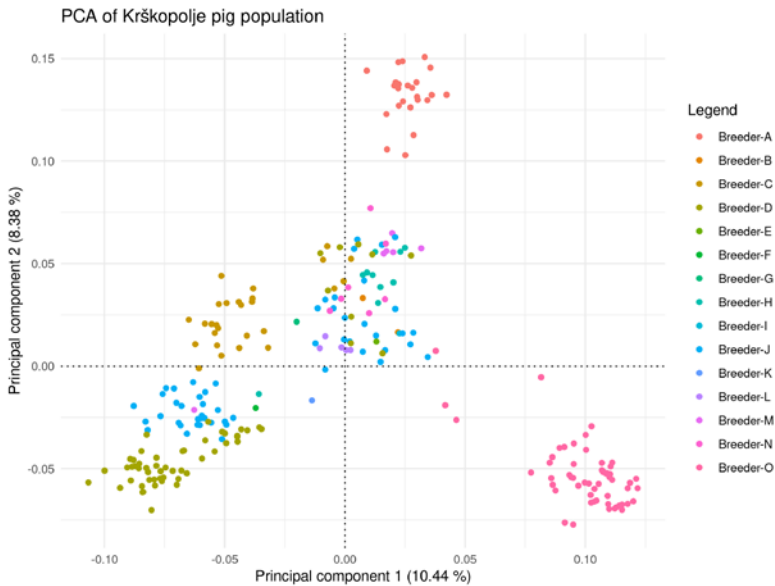


Figure 2. Genetic structure of different Krškopolje pig animals originating from 15 different breeders

Conclusions

The present study confirmed that the incidence of the mutant *RYRI* allele in Krškopolje pig is still relatively high, and therefore initiatives are needed to

eliminate it, but with prudence not to jeopardise breed's genetic diversity. In addition, the segregation of the *PRKAG3* Ile249Val and *MC4R* c.892G>A alleles in the Krškopolje pig represents an opportunity for further association studies and/or possible practical applications in the breeding program. Maintenance of relatively high heterozygosity should also be considered in the development of conservation and breeding strategies.

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References

- BATOREK-LUKAČ N., TOMAŽIN U., ŠKRLEP M., KASTELIC A., POKLUKAR K., ČANDEK-POTOKAR M. Krškopoljski prašič (Krškopolje pig). In: European local pig breeds – diversity and performance: A study of project TREASURE. Intech Open, London, UK. 141-154.
- BORDONARO S., CHESSARI G., MASTRANGELO S., SENCZUK G., CHESSA S., CASTIGLIONI B., et al. (2023): Genome-wide population structure, homozygosity, and heterozygosity patterns of Nero Siciliano pig in the framework of Italian and cosmopolitan breeds. *Animal Genetics*, 00, 1-15.
- CIOBANU D., BASTIAANSEN J., MALEK M., HELM J., WOOLLARD J., PLASTOW G., et al. (2001): Evidence for new alleles in the protein kinase adenosine monophosphate-activated gamma(3)-subunit gene associated with low glycogen content in pig skeletal muscle and improved meat quality. *Genetics*, 159, 3, 1151-1162.
- ČANDEK-POTOKAR M., NIETO LINAN R.M. (2019): European local pig breeds – diversity and performance: A study of project TREASURE. Intech Open, London, UK. pp303.
- ENFÄLT A.-C., VON SETH G., JOSELL A., LINDAHL G., HEDEBROVELANDER I., BRAUNSCHWEIG M., et al. (2006): Effects of a second mutant allele (V199I) at the *PRKAG3* (RN) locus on carcass composition in pigs. *Livestock Science*, 99, 2-3, 131-139.
- FUJII J., OTSU K., ZORZATO F., DE LEON S., KHANNA V.K., WEILER J.E., et al. (1991): Identification of a mutation in porcine ryanodine receptor associated with malignant hyperthermia. *Science*, 253, 5018, 448-451.
- HERRERO-MEDRANO J.M., MEGENS H.-J., GROENEN M.A.M., BOSSE M., PÉREZ-ENCISO M., CROOIJMANS R.P.M.A. (2014): Whole-genome sequence analysis reveals differences in population management and selection of Europe low-input pig breeds. *BMC Genomics*, 16, 15, 601.

- KIM K.S., LARSEN N., SHORT T., PLASTOW G., ROTHSCCHILD M.F. (2000): A missense variant of the porcine melanocortin-4 receptor (MC4R) gene is associated with fatness, growth, and feed intake traits. *Mammalian Genome*, 11, 131-135.
- MERCAT M.-J., AMARAL A., BOZZI R., ČANDEK-POTOKAR M., FERNANDES P., GUTIERREZ VALLEJOS J., et al. (2022): Stakeholders' perception of the local breeds' sector in six European countries – a survey by the GERO-NIMO project. Book of abstracts of the “XI. International Symposium on the Mediterranean pig”, October 11-14, Vodice, Croatia, 87-88.
- MILAN D., JEON J.T., LOOFT C., AMARGER V., ROBIC A., THELANDER M., et al. (2000): A mutation in PRKAG3 associated with excess glycogen content in pig skeletal muscle, *Science*, 288, 5469, 1248-1251.
- MUÑOZ M., BOZZI R., GARCÍA F., NÚÑEZ Y., GERACI C., CROVETTI A., et al. (2018): Diversity across major and candidate genes in European local pig breeds. *Plos One*, 13, 11, e0207475.
- MUÑOZ M., BOZZI R., GARCÍA-CASCO J., NÚÑEZ Y., RIBANI A., FRANCI O., et al. (2019): Genomic diversity, linkage disequilibrium and selection signatures in European local pig breeds assessed with a high density SNP chip. *Scientific reports*, 9, 13546.
- PIÓRKOWSKA K., TYRA M., ROGOZ M., ROPKA-MOLIK K., OCZKOWICZ M., RÓZYCKI M. (2010): Association of the melanocortin-4-receptor (MC4R) with feed intake, growth, fatness and carcass composition in pigs raised in Poland. *Meat Science*, 85, 2, 297-301.
- POKLUKAR K., ČANDEK-POTOKAR M., BATOREK-LUKAČ N., ŠKRLEP M. (2023): Biochemical and gene expression differences associated with higher fat deposition in Krškopolje pigs in comparison with lean hybrid pigs. *Livestock Science*, 272, 105247.
- PURCEL S., NEALE B., TODD-BROWN K., THOMAS L., FERREIRA M.A.R., BENDER D., et al. (2007): PLINK: A Tool Set for Whole-Genome Association and Population-Based Linkage Analyses. *American Journal of Human Genetics*, 81, 3, 559-557.
- RAUW W.M., KANIS E., NOORDHUITEN-STASSEN E.N., GROMMERS F.J. Undesirable side effects of selection for high production efficiency in farm animals: a review. *Livestock Production Science*, 56, 15-33.
- SALMI B., TREFAN L., BLOOM-HANSEN J., BIDANEL J.P., COESCHL-WILSON A.B., LARZUL C. (2010): Meta-analysis of the effect of the halothane gene on 6 variables of pig meat quality and on carcass leanness. *Journal of Animal Science*, 88, 9, 2841-2855.
- TOMAŽIN U., POKLUKAR K., ŠKRLEP M., BATOREK LUKAČ N., ČANDEK-POTOKAR M. (2021): The effect of *RYR1* gene on meat quality in autochthonous breed Krškopolje pig. Proceedings of the: “13th International

symposium modern trends in livestock production”, October 6-8, Belgrade, Serbia, 561-571.

TRIBOUT T., CARITEZ J.C., GRUAND J., BOUFFAUD M., GUILLOUET P., BILLON Y. et al. (2010): Estimation of genetic trends in French Large White pigs from 1977 to 1998 for growth and carcass traits using frozen semen. *Journal of Animal Science*, 88, 1856-1867.