Is *KIT* Locus Polymorphism rs328592739 Related to White Belt Phenotype in Krškopolje Pig?

Jernej OGOREVC [1], Minja ZORC [1], Martin ŠKRLEP [2], Riccardo BOZZI [3], Matthias PETIG [4], Luca FONTANESI [5], Marjeta ČANDEK-POTOKAR [2], Peter DOVČ [1(✉)]

Summary

Krškopolje pig is a local Slovenian breed with black coat and white belt of variable width over the shoulders. The breed experienced serious bottle neck effect in the middle of the 20th century followed by the introgression of different breeds, some of them with the goal to preserve belted phenotype. The belt allele was assigned to the KIT proto-oncogene receptor tyrosine kinase (*KIT*) gene. A number of single nucleotide polymorphisms (SNPs) and structural variations on of the *KIT* locus were observed. The synonymous SNP rs328592739 in the *KIT* gene was reported as a marker for distinguishing meat of belted Cinta Senese pigs from meat of non-belted breeds. In the present study, the SNP rs328592739 in Krškopolje, Cinta Senese and Swäbisch-Hall belted pigs has been genotyped using PCR-RFLP approach. In Krškopolje pig the region surrounding SNP rs328592739 was also sequenced using Sanger sequencing. In addition, publicly available whole-genome sequencing data for pigs of 12 different breeds and wild boar were analysed to obtain the sequence of *KIT* locus and its surrounding region in belted and non-belted pig breeds. The results show that SNP rs328592739 is not associated with white belt phenotype across different pig breeds. However, the analysis revealed structural variations within the *KIT* locus which may be associated with belted phenotype in pigs.

Key words

coat colour, *KIT*, *Sus scrofa*, rs328592739, white belt phenotype

[1] Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Groblje 3, SI-1230 Domžale, Slovenia
✉ e-mail: peter.dovc@bf.uni-lj.si
[2] Agricultural Institute of Slovenia, Hacquetova ulica 17, SI-1000 Ljubljana, Slovenia
[3] Department of Agricultural Biotechnology, Animal Science Section, Via delle Cascine 5, 50144 Firenze, Italy
[4] Bäuerliche Erzeugergemeinschaft Schwäbisch Hall w.V., Raiffeisenstraße 18, 74523 Schwäbisch Hall, Germany
[5] Department of Agricultural and Food Sciences, Division of Animal Sciences, Laboratory of Livestock Genomics, University of Bologna, Bologna, Italy, Centre for Genome Biology, University of Bologna, Bologna, Italy

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Introduction

Krškopolje pig is a local Slovenian breed with black coat and distinctive white belt of variable width over the shoulders. The belt phenotype is, in addition to Krškopolje pig, a characteristics of several European pig breeds, including Hampshire, Cinta Senese, Angler Sattelschwein, Swäbisch-Hall, Wessex, Essex, Hannover-Braunschweig, Basque, Limousin and Bavarian Landschwein, as well as some Chinese pig breeds, such as Bamaxiang, Dongshan, Ganxi, Jinhua, Rongchang, Shaziling, and Tongcheng. The studies on the coat colour genetics were initiated shortly after rediscovery of Mendel's laws in 1900, mainly due to the fact that coat colour is a trait which is easy to score and often shows higher diversity in domestic animals than in natural populations. In general, mammalian pigmentation has long served as a model system to study molecular mechanisms that shape the phenotype (Hubbard et al., 2010).

The dominant inheritance of the white coat in pig and inheritance of white belt phenotype in Hampshire were first described by Spillman (Spillman 1906, 1907). Segregation analysis using an intercross between belted (Hampshire) and non-belted (Piétrain) pigs assigned the belt phenotype to KIT proto-oncogene receptor tyrosine kinase gene (*KIT*), suggesting that the white belt phenotype might be caused by mutation within the regulatory element which affects the *KIT* gene expression during development (Giuffra et al., 1999). A 4.3-kb duplication located ~100 kb upstream of *KIT* locus in Hampshire pigs has been assumed as a causative mutation for the belted phenotype (Rubin et al., 2012). Several *KIT* gene haplotypes were identified in different pig breeds. In two belted pig breeds (Hampshire and Cinta Senese) a selection signature was evident at the *KIT* locus (Fontanesi et al., 2010). The synonymous SNP in exon 18 of the *KIT* gene (rs328592739) was found to be informative for distinguishing meat of Cinta Senese pigs from meat of the non-belted pigs (Fontanesi et al., 2016), indicating that T allele could be considered as "belted allele" in Cinta Senese. In Chinese pig breeds, the analysis based on the SNP array data of belted and non-belted breeds revealed an additional gene, Endothelin Receptor Type B (*EDNRB*) gene as a candidate for the white belt phenotype (Ai et al., 2013).

KIT gene encodes tyrosine kinase receptor and spans over 89 kb of genomic DNA, consisting of 21 exons that range in size from 100 to 200 bp (Yarden et al., 1987, Vandenbark et al., 1992). KIT is a key regulatory molecule involved in the development and the homeostasis of hematologic, mast, germ and melanocytic cell systems (Ronnstrand 2004). During the embryonic development, KIT is involved in the process of melanocyte colonization of the developing epidermis (Besmer et al., 1993). A number of mutations in the *KIT* gene associated with pigmentation have been reported in humans, mice, pigs, cattle, horses, cats and dogs. In humans, mutations in KIT gene cause a condition called piebaldism, clinically manifested as depigmentation of the ventral chest and abdomen with white fare flock of hair (OMIM). This pattern suggests that the developing melanocytes lack the capacity to migrate to locations distant from the neural crest (Grichnik 2006). Associated with human piebaldism, 32 missense mutations, 17 deletions,http://onlinelibrary.wiley.com/doi/10.1111/j.1346-8138.2012.01583.x/full - b31 four insertions,http://onlinelibrary.wiley.com/doi/10.1111/j.1346-8138.2012.01583.x/full - b31 seven nucleotide splice-site mutations,http://onlinelibrary.wiley.com/doi/10.1111/j.1346-8138.2012.01583.x/full - b31 two nonsense mutations and one pericentric chromosomal inversion have been identified near or on the *KIT* locus (Oiso et al., 2013). In mice, there are variants of the *KIT* gene that cause the belt pattern in heterozygous condition but a spotted or patch appearance in homozygotes (Kluppel et al., 1997).

Material and methods

Animals and DNA extraction

DNA was extracted from ear tissues collected from 38 pigs of Krškopolje breed, nine pigs of Swäbisch-Hall breed and from hair of five pigs of Cinta Senese breed. The DNA extraction was performed using Isolate II Genomic DNA Kit (Bioline) according to manufacturer's instructions.

PCR-RFLP analysis and sequencing

Polymerase chain reaction (PCR) was performed to screen for C/T SNP (rs328592739) in the amplified 230 bp fragment of the KIT locus, using forward: 5'- CAGTCAGGGTCATCCAAGGT -3' and reverse: 5'- AGGACCAGACATCGCTTTCA -3' primers, designed with Primer3 software. The amplification reactions were performed on ABI 2720 thermal cycler as follows: 5 min initial denaturation at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and synthesis at 72 °C for 20 s, and the final elongation step at 72 °C for 5 minutes. The reaction volume was 20 µl and contained 1 x PCR buffer, 0.75 µM primers, 150 µM dNTPs, 1.2 mM $MgCl_2$, 0.5 U DNA Taq polymerase (Thermo Fisher Scientific), and approximately 50-200 ng template DNA.

The PCR product was digested using restriction endonuclease *Dde*I (R0751S, New England Biolabs) to obtain fragments of 180 and 50 bp in case of T allele and 230 bp in case of C allele. The restriction site and allele sizes were predicted using NEBcutter V2.0 software. The restriction reaction consisted of 10 µl PCR product, 1.5 µl restriction buffer, 3.25 µl H_2O, 0.25 µl (2.5U) of the enzyme, and was incubated for 2 h at 37 °C. Fragments after restriction were analysed on 2.5 % agarose gel run at 100 V for 30 minutes, stained with ethidium bromide and visualized on U:Genius3 Gel Documentation System, Syngene.

Additionally, PCR products were sequenced to confirm the PCR/RFLP results. Briefly, a 254 bp fragment around the SNP was amplified, using forward: 5'- AGGACTTTGTGAGATGCCCG -3' and reverse: 5'- GCCTTTGGCAAGGTGCATTT -3' primers, designed using Primer3 software. Amplified fragments were treated with exonuclease I (ExoI) and alkaline phosphatase (FastAP) (both Thermo Fisher Scientific) for 15 min at 37 °C. Treated amplicons were used for Big Dye v3.1 sequencing reaction (Thermo Fisher Scientific) using forward and reverse primers. The fragments were purified using EDTA and ethanol, resuspended in formamide and loaded on an ABI3100 sequencer (Applied Biosystems).

Whole-genome sequencing data processing

Whole-genome sequencing data were downloaded from the European Nucleotide Archive (ENA) under accession codes PRJEB9922 (Frantz et al., 2015) and PRJNA239399 (Molnár et al., 2014). Accession numbers of downloaded sequences are: WB28F31 (Wild boar - Italy), AS01F01, AS01F09 (Angler

Sattelschwein), BK01F10, BK01M20 (Berkshire), NI01U07 (Black Iberian), BS01F10 (British Saddleback), LE01F25 (Large White), CA01F14 (Calabrese), CT01F13, CT01M12 (Casertana), CS01F02 (Cinta Senese), HA20U01, HA20U02 (Hampshire), SRR1178916, SRR1178923, SRR1178924, SRR1178925, MA01F18, MA01F20 (Mangalica), NS01F05 (Nera Siciliana), JI01U08 (Jinhua). Reads were downloaded in FASTQ format and uploaded to the local instance of Galaxy (Afgan et al., 2016).

FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to evaluate the quality of sequences before and after trimming process. Trimming was performed using Trimmomatic (Bolger et al., 2014). Low quality bases (Phred score < 20), and short reads (< 30 bp) were removed. The clean reads were mapped against the reference genome (Sscrofa11.1) using BWA-MEM (Li and Durbin 2010). Golden Helix GenomeBrowse tool was used for visualisation of resulting alignments and Integrative Genomics Viewer (IGV) (Robinson et al., 2011) to obtain the consensus sequence of genomic region of interest.

Results

SNP rs328592739 and its flanking region

PCR/RFLP analysis showed that all 38 tested Krškopolje pigs were homozygous for the wild type allele C at the rs328592739 (C > T) position. Seven pigs of Schwäbisch-Hall breed were homozygous for the wild type allele C and 2 of them were heterozygous. Five animals of the Cinta Senese breed were homozygous for the T allele.

From the publicly available whole-genome sequencing data for 21 pigs of 12 different breeds and one wild boar we identified

Table 1. rs328592739 genotype in 22 pigs of different breeds determined from publicly available whole-genome sequencing data

Breed and sample names	Coat colour characteristic for the breed	rs328592739 genotype
Wild boar - Italy (WB28F31)	coat colour ranges in colour from black to brownish-red to white	CC
Angler Sattelschwein (AS01F01, AS01F09)	white-belted	CT
		CC
Berkshire (BK01F10, BK01M20)	black with white points on the nose, tail, and limbs	CC
		CC
Black Iberian (NI01U07)	black (some animals are white-belted)	CC
British Saddleback (BS01F10)	white-belted	CC
Large White (LE01F25)	White	CC
Calabrese (CA01F14)	black (some animals are spotted or have white socks)	CC
Casertana (CT01F13, CT01M12)	dark coat, often getting to purple brown	CC
		CC
Cinta Senese (CS01F02)	white-belted	TT
Hampshire (HA20U01, HA20U02)	white-belted	TT
		TT
Mangalica (SRR1178916, SRR1178923, SRR1178924, SRR1178925, MA01F18, MA01F20)	black with a blond belly and feet	CT
	blond	TT
		TT
	dark or light shade of brownish red	CT
	swallow belly, blond or red	TT
		CC
Nera Siciliana (NS01F05)	completely black	CC
Jinhua (JI01U08)	white-belted	CC

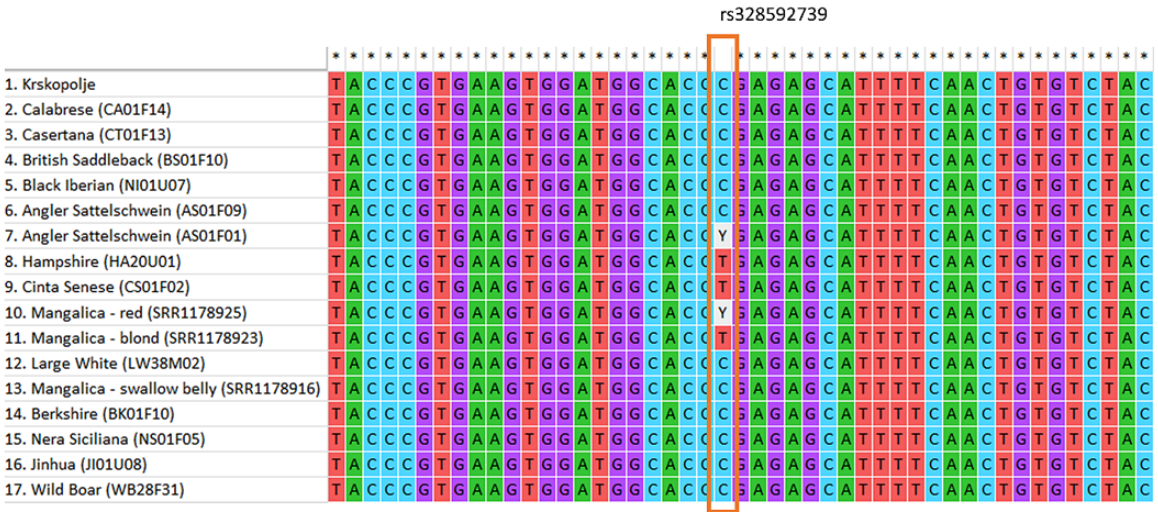

Figure 1. Alignment of the sequence of the region within exon 18 of the *KIT* gene including SNP rs328592739. The IUPAC ambiguity code Y means there was a heterozygous sample (C/T).

13 homozygotes for allele C, six homozygotes for allele T and three heterozygotes at the locus rs328592739 (Table 1).

The genotyping results for Krškopolje pigs were confirmed by Sanger sequencing. We have aligned four sequences of Krškopolje pigs with the consensus sequences of the same genomic region obtained from the publicly available whole-genome sequencing data for 22 animals (Figure 1). The sequences were aligned in MEGA (http://www.megasoftware.net) using Muscle algorithm.

Structural variations in flanking regions of *KIT* gene

Analysis of the wider genomic region containing *KIT* locus revealed an increased depth of sequence coverage in two regions close to the *KIT* locus (Figure 2). The first region with the increased depth of coverage appears in the belted and white animals only and is located on the chromosome 8 between 41290130 and 41294451 (Sscrofa 11.1 assembly). The length of the region

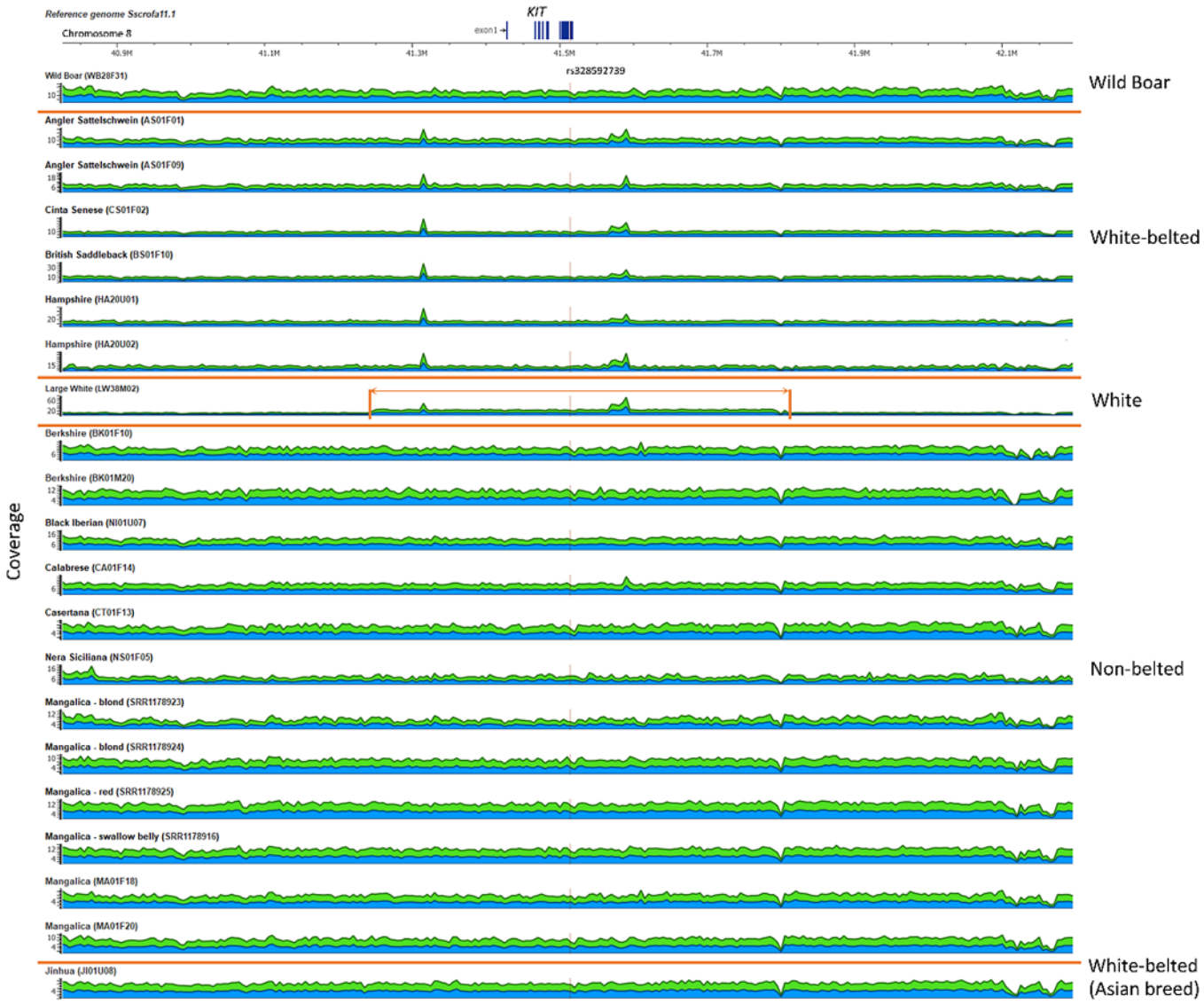

Figure 2. Sequencing depth of coverage for the *KIT* gene and its flanking regions. Increased coverage in the flanking regions of the *KIT* gene indicates the presence of duplications. Increased coverage in upstream region of the gene *KIT* is present in belted and white pigs included in the analysis. Increased coverage of large region which includes entire *KIT* gene in White Large pig indicates the duplication of the region (arrows show the beginning and the end of the duplicated region), while the reference genome originates from a Duroc pig with a single copy of *KIT*.

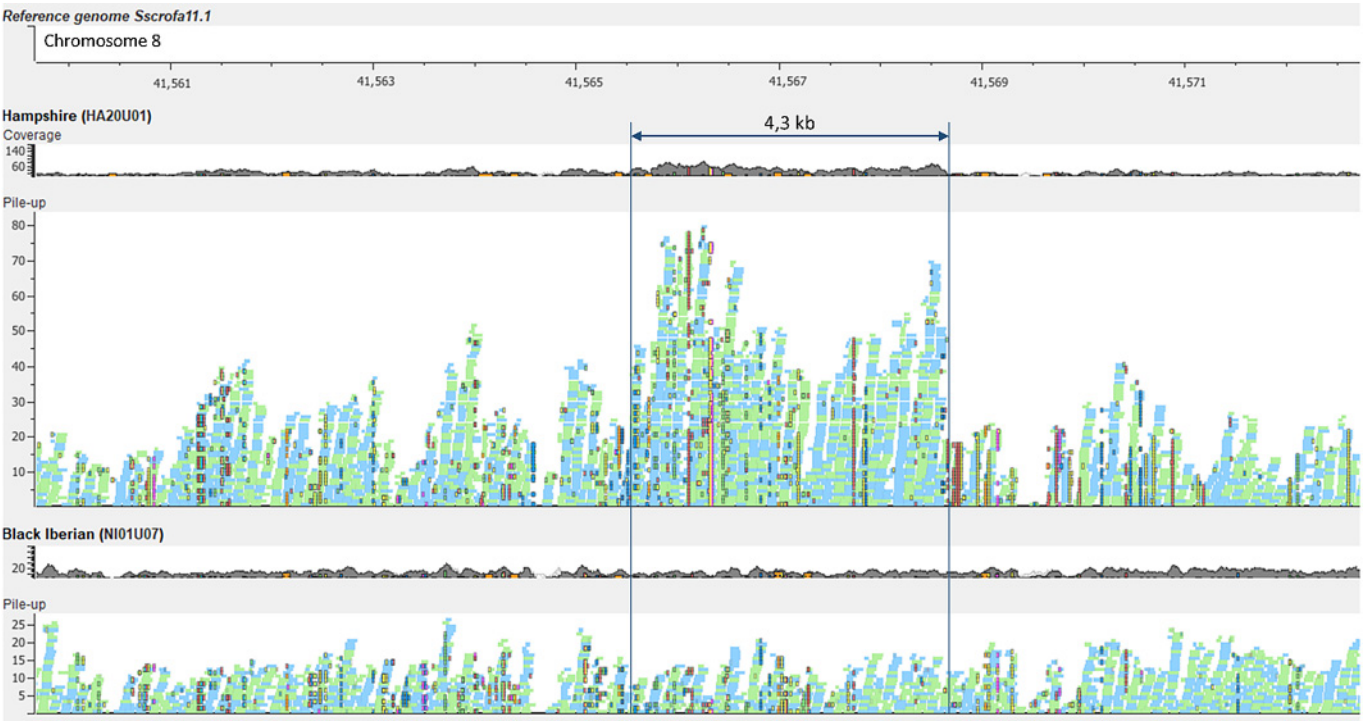

Figure 3. Increased depth of coverage in the region 4.3 kb of length 112.2 kb upstream of the *KIT* gene.

Figure 4. Belted phenotype in pigs of A) Krškopolje pig, B) Schwäbisch-Hall breed rs328592739 genotype C/C and B) Schwäbisch-Hall breed rs328592739 genotype C/T

is approximately 4.3 kb and is located 112.2 kb upstream of the *KIT* locus (Figure 3). The second region, located between g.41563770 and g.41568720, is approximately 5 kb long and is located 161.4 kb downstream of *KIT*. However, in Large White, the entire genomic region covering the wider area around the *KIT* locus is duplicated (Figure 2).

Discussion

Our data show that the T allele on analysed SNP (rs328592739 (C > T)) is not associated with the belted phenotype in Krškopolje and Schwäbisch-Hall pig. Krškopolje breed was graded up, due to inbreeding problems, with different breeds, among others with Angler Sattelschwein and Wessex Saddleback. For Angler Sattelschwein we found two sequences with CT and CC genotypes (Table 1). Contrary to Fontanesi et al., (2016) who reported high frequency of the T allele in belted Cinta Senese and Hampshire breeds, and suggested that T (or "belted") allele on this locus could be used to distinguish meat of Cinta Senese from meat of other non-belted pig breeds from the region in pork products, we found only the C allele in Krškopolje pig which also has belted phenotype.

In addition, we analysed publicly available *KIT* sequences from different breeds to compare the rs328592739 SNP in belted and non-belted pig breeds. European wild boar and most of the non-belted breeds have CC genotype (Fontanesi et al., 2016). Interestingly, some belted breeds like German Angler Sattelschwein and Chinese Jinhua also have CC genotype but all three genotypes can be found in non-belted Mangalica pigs. Based on our results, including Krškopolje pigs and publicly available sequences, we suggest that the SNP rs328592739 (C > T) is not a genetic marker for the belted phenotype, in spite of the fact that TT genotype is predominant in belted Hamsphire and Cinta Senese breeds.

The analysis of whole-genome sequencing data for pigs of different breeds indicates the presence of structural variations in the vicinity of the *KIT* locus which supports the results of the study where the variations of the same length at exactly the same genomic positions were reported in Hampshire breed as a representative for belted breeds (Rubin et al., 2012). In agreement with earlier studies which argued that belted phenotype in pigs might be caused by regulatory mutation of the *KIT* gene (Giuffra et al., 1999), we identified the structural variation in the upstream region of the *KIT* gene in the genomes of belted and white breeds only (Figure 2). The variation overlaps with one of the most conserved noncoding regions upstream of *KIT* locus and might constitute a regulatory element (Rubin et al., 2012). This suggestion is in agreement with general opinion that noncoding mutations and structural variations contributed significantly to the evolution of phenotypic diversity in domestic animals (Andersson 2012).

The belted phenotype, describing pigs with black heads and hips separated by white belt across their bodies, is very variable in pigs. The belts are differently extensive, can totally encircle the body and can include both front legs (Figure 4). Pigs with the dominant white coat colour phenotype carry a duplication of the *KIT* gene encoding the mast/stem cell growth factor receptor (Johansson Moller et al., 1996). The wide genomic duplication shown in Figure 3 has also been identified in animals with dominant white phenotype by Rubin et al., 2012. The phenotypic effect of copy number variation (CNV) affecting the regulatory element at *KIT* locus in dominant white pig breeds may become stronger with CNV expansion – an effect also demonstrated for greying with age in horses (Sundstrom et al., 2012).

Conclusion

Based on our results, including Krškopolje pig and publicly available sequences from 12 pig breeds, we suggest that SNP (rs328592739 (C > T)) is not a genetic marker for the belted phenotype across the breeds, although it seems to be associated with the belted phenotype in Cinta Senese and Hampshire breeds. The alignment of genomic sequences of publicly available pig breeds revealed three structural mutations with higher sequence coverage suggesting genomic duplications approximately 112 kb upstream and 160 kb downstream of the *KIT* locus and the duplication of the region including entire *KIT* gene. The 4.3 kb duplication located upstream of the *KIT* locus seems to be associated with belted phenotype in numerous pig breeds.

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