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Variability of the *IGF2 locus* in the Suino Nero Lucano pig population and its effects on meat quality

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Abstract. The aim of this study was to analyse the polymorphisms in the two promoter regions, P1 and P2, of the porcine Insulin-like Growth Factor 2 (*IGF2*) gene and to investigate the effect of *IGF2* genotypes on meat quality traits in the Italian autochthonous Suino Nero Lucano pig. Three polymorphic sites were analysed and only two of the eight potential haplotypes were observed in the Suino Nero Lucano pig population: A haplotype (–366A ▬ –225G ▬ –182C), and B haplotype (–366G ▬ –225C ▬ –182T). Muscle mass and meat quality characteristics were analysed in 30 castrated pigs (10 for each of the three *IGF2* genotypes: A/A, A/B, and B/B). According to the results, B/B animals, at the same carcass weight, showed the highest *Longissimus lumborum* and *Psoas* weight ($P < 0.05$), whereas A/A animals showed a higher intramuscular fat percentage and lower Warner–Bratzler shear force, drip loss, and polyunsaturated fatty acids content. Meat from B/B animals showed also a higher L^* value and myoglobin and deoxymyoglobin percentage compared with meat from A/A ones ($P < 0.05$).

Additional keywords: fatty acid, *IGF2* polymorphism, Italian autochthonous pig, meat colour.

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Introduction

In recent years particular interest has been shown in meat obtained from ancient autochthonous pig breeds. The Suino Nero Lucano (SNL) pig is an autochthonous breed of Southern Italy (Basilicata region) that ~10 years ago was in danger of extinction, whereas today shows a relatively safe consistency (~3000 individuals). This breed is strongly appreciated for the high quality of its raw and dry-cured products obtained through traditional methods (Perna *et al*. 2015*a*, 2015*b*). It is known that the Insulin-like Growth Factor 2 gene (*IGF2*) is an important regulator of growth, development and differentiation of the skeletal muscle (Brown *et al*. 2009; Duan *et al*. 2010; Livingstone and Borai 2014). This gene is a member of a gene family producing structurally related polypeptides, the *IGF* family, which includes two ligands (*IGF1* and *IGF2*), three cell membrane receptors, and several other associated binding proteins and regulators (Denley *et al*. 2005). The *IGF2* polypeptides are important for prenatal and postnatal growth. In fact, they stimulate both differentiation and proliferation of muscle cells (Kokta *et al*. 2004), they are significant for muscle regeneration after injury (Devaney *et al*. 2007), and they are involved in myofibre hypertrophy (Armand *et al*. 2004). As the genetic variability of the *IGF2* gene in pig is associated with strong effects on both leanness and fat deposition, this gene is considered a quantitative trait locus (QTL) for meat production and slaughter traits (Jeon *et al*. 1999; Nezer *et al*. 1999). In pig, as in man and sheep, the *IGF2* gene is organised in 10 exons (one named 4b) of which only the last three encode for a pre-pro *IGF2* protein of 180aa. The transcription is under the control of four promoter regions (numbered from P1 to P4), which act in a tissue-specific and development-dependent manner (Ohlsen *et al*. 1994; Mineo *et al*. 2000; Amarger *et al*. 2002). Several *IGF2* polymorphisms were identified in the pig (Van Laere *et al*. 2003) none of which was found in the coding sequence of the gene (Nezer *et al*. 2003). In particular, the transition G→A located at the nucleotide 3072 of *IGF2* intron 3 is tightly associated with muscle growth, fat deposition and size of the heart in pig (Van Laere *et al*. 2003). The phenotypic effect of the G/A substitution was reported in several pig crosses (Van den Maagdenberg *et al*. 2008; Oczkowicz *et al*. 2009; Burgos *et al*. 2012) and it was associated with: backfat thickness, muscle leanness, fatness, growth, and carcass traits. In general, breeds selected for a high muscularity (e.g. Duroc and Pietrain) are characterised by a high frequency of the A allele. On the contrary, autochthonous breeds, never selected for muscularity, such as Iberian, Casertana, Cinta Senese and Nero Siciliano, are characterised by a high frequency of the G allele (Carrodeguas *et al*. 2005; Fontanesi *et al*. 2010). Aslan *et al*. (2012) identified other six SNP in the *IGF2* promoters, two in P1 and four in P2, and they associated different effects of the observed haplotypes with fat deposition and marbling.

The aim of the present study was to analyse the polymorphisms in the two promoter regions, P1 and P2, of the porcine *IGF2* gene and to investigate the effects of the *IGF2* genotypes on the meat quality traits in the SNL pig.

Materials and methods

Animals

IGF2 gene polymorphism was analysed in 166 purebred SNL castrated male pigs randomly selected from different farms of the province of Potenza (Southern Italy). After identification of the *IGF2* genotypes ($n = 3$), 10 individuals for each of the three genotypes, were randomly selected at ~90 days of age, located in the same farm, and raised under a semi-wild system. All pigs were fed the same diet until their slaughter. Feed and water were provided *ad libitum*. The composition of the diet was: corn 20%, field beans 25%, barley 20%, bran and residues from processing of cereals 15%, administered as grains mixture in the form of crushed, in addition to pasture. Pigs were slaughtered at ~540 days when they reached the weight of ~140 kg. *Longissimus lumborum* (LL) and *Psoas* (P) muscles from the right side of the carcass were excised (sample cut) at 24 h post mortem. The sample cut was made perpendicular to the long axis of the carcass at the cranial end of the 2nd lumbar vertebra and the caudal end of the 5th lumbar vertebra. The weight of LL and P muscles (obtained from sample cut) was recorded, and LL samples were vacuum packed and stored at −20°C until analysis.

IGF2 genotyping

DNA from 100 to 120 mg of meat was extracted using RapidPure DNA Tissue Kit (Life Science MPbiomedicals, Solon, OH, USA) following guideline procedures. A 657-bp fragment of the IGF2 gene P1 promoter was amplified using the following primers: forward P1 5′CCTCTCTGCTCTCGCCACATC3′; reverse P1: 5′CACTCATCCCAGCCGCCTAC3′. A 480-bp fragment of the IGF2 gene P2 promoter was amplified from the same DNA samples using the following primers: forward P2 5′CAGGTTGC CCCCAGTTTAGAC3′; reverse P2: 5′CTGCGTCGGAGTGG AGAA3′. Both PCR reactions were carried out in a final volume of 50 µL containing: 200 ng DNA, 1X buffer, 3 mM $MgCl_2$, 400 µM dNTPs, 20 pmol each primers, 2.5U *Taq* DNA polymerase (Promega, Madison, WI, USA). The PCR reactions were accomplished as follow: denaturation 45 s at 95°C; annealing and extension 1 min at 78°C for 31 cycles for P1 amplification, and denaturation 45 s at 95°C; annealing 45 s at 65°C extension 45 s at 72°C for 36 cycles for P2 amplification. The 657-bp PCR products were digested with *Pst*I restriction endonuclease to analyse the polymorphisms G > A at –366 (position 3530 on GenBank Ref. AY242098.1) *locus*. The 480-bp PCR products were digested separately with Eco0109 I, *Mbo*I and DraIII-HF restriction endonucleases to analyse the polymorphisms C > G at –225, G > C at –209 and *t* > C at –182 loci (positions 19066, 19082, and 19109 on GenBank Ref. AY242098.1), respectively. The PCR–restriction fragment length polymorphism products were analysed on 2% agarose gel stained with ethidium bromide. Linkage disequilibrium among the polymorphic sites was estimated using HAPLOVIEW software version 4.2 (http://www.broadinstitute.org/haploview/haploview, accessed 1 June 2017).

Chemical composition and rheological parameters

Dry matter (DM), protein, intramuscular fat (IMF), and ash contents of LL muscle were determined according to AOAC (1995) methods. DM was determined by oven drying method at 105°C until constant weight (method 950.46), protein by Kjeldahl method (method 990.03) using a 6.25 factor to convert the nitrogen content into total protein, IMF by Soxhlet extraction (method 920.39), and ash by using a muffle furnace for 12 h at 550°C (method 920.153). Haem iron content was determined according to the method described by Hornsey (1956). The water-holding capacity (WHC) of LL muscle at 24 h post mortem, measured as Percent Drip Loss, was scored on the basis of the bag method (Honikel 1998). Briefly, ~80 g of meat were accurately weighed, suspended in an inflated plastic bag, held at 4°C and reweighed 48 h later. Drip loss was calculated as a percentage of lost weight based on the starting weight of the sample. All samples were analysed in duplicate. Warner–Bratzler shear force (WBS) was determined by Instron Universal Testing Machine (Model 1011, Instron Corp., USA) equipped with a Warner–Bratzler shearing device. Six cores (Ø 2.54 cm diameter) were removed from each steak parallel to the longitudinal orientation of muscle fibres. Samples were sheared perpendicular to the long axis of the core. WBS was determined according to the peak in the force deformation curve, and its unit was expressed in Newton.

Lipid analyses

The IMF of the LL muscle samples was extracted using chloroform/methanol (1 : 2) according to Folch *et al.* (1957), and fatty acid methyl esters (FAME) were prepared according to the ISO (1978) method. Analysis was performed using a Varian 3400 gas chromatograph (Varian, Turin, Italy), equipped with a split-splitless injector, a TR-FAME capillary column (120 m × 0.25 mm i.d. × 0.25-mm film thickness; Thermo Fisher Scientific, Waltham, MA, USA), a flame ionisation detector and a Galaxie™ Chromatography Software (Varian, Inc., Palo Alto, CA, USA) for chromatogram acquisition and data reporting. Helium was used as carrier gas, and the injector and detector temperatures were 250°C and 260°C respectively. The oven temperature program was 140°C for 5 min then increasing at 4°C/min up to 240°C where it was maintained for 15 min. Individual FAME were identified by comparing their retention times with those of the corresponding pure standards (Sigma-Aldrich, Milan, Italy). Quantitative analysis was obtained by peak area integration using the Galaxie™ Chromatography Data System Version 1.9.3.2 software (Varian, Inc.) and results were expressed as percentage of the total analysed fatty acids.

Instrumental colour measurement

Instrumental colour (CIE L^*, a^*, b^*) was measured using a MINOLTA Chromameter CR-300 (Minolta Camera Corp., Meter Division, Ramsey, NJ, USA) equipped with a D65 illuminant, the 10° Observer and zero and white calibration. The measuring head has an 8-mm-diameter measuring area. The following colour coordinates were determined: lightness

(*L**), redness (*a**, ± red-green) and yellowness (*b**, ± yellow-blue). Before each measurement the equipment was standardised against a white tile. The meat colour was assessed 24 h post mortem. The measurements were performed on surface of LL muscle samples and the analysis was performed in quadruplicate.

Myoglobin analyses

The relative contents of myoglobin (Mb), oxymyoglobin (MbO_2) and metmyoglobin (MetMb) were calculated, in LL muscle samples, from the reflectance curve according to Krzywicki (1982) using the wavelength of 710 nm (the highest available from the instrument) instead of 730 nm.

Statistical analyses

Data were analysed according to the following linear model (SAS Institute 1996):

$$y_{ik} = \mu + \alpha_i + \varepsilon_{ik}$$

where y_{ik} is the observation; μ is the overall mean; α_i is the fixed effect of the *i*th *IGF2* genotype (*W*) ($i = 1, 2$); and ε_{ik} is the random error. Before setting the values, expressed in percentage terms, they were subjected to arcsine transformation. Differences between means at the 95% ($P < 0.05$) confidence level were considered statistically significant.

Results and discussion

IGF2 haplotypes

All DNA samples showed a 657-bp fragment for the P1 promoter amplification and a 480-bp fragment for the P2 promoter amplification. The *Pst*I digestion of the 657-bp PCR products yielded three electrophoretic patterns: a two bands pattern (405 bp and 252 bp) for the homozygous –366 A/A individuals; a single band pattern (uncut 657 bp) for the homozygous –366 G/G individuals; and a three bands pattern (657 bp, 405 bp and 252 bp) for the heterozygous –366 A/G individuals. The digestion of the 480-bp PCR products with Eco0109 I restriction enzyme allowed to identify genotypes at the –225C > G locus: 480-bp uncut fragment for G/G homozygotes; 352-bp and 128-bp fragments for C/C homozygotes, and 480-bp, 352-bp and 128-bp fragments for G/C heterozygotes. The digestion with DraIII-HF restriction enzyme allowed to distinguish genotypes at the –182*T* > C locus: 480-bp uncut fragment for C/C homozygotes; 396-bp and 84-bp fragments for T/T homozygotes, and 480-bp, 396bp and 84-bp fragments for C/T heterozygotes. As the 480-bp fragment digestion with *Mbo*I showed two fragments (370 bp and 110 bp) for all individuals, the –209G > C locus is monomorphic for the G allele in the analysed animals. Genotype and allele frequencies at IGF2 –366G > A, –225C > G and –182*T* > C loci are reported in Table 1. According to the results, the genotype distributions at the three loci are not in Hardy–Weinberg equilibrium. This result is determined by an excess of homozygous individuals due to inbreeding effects ($F = 0.26$) consequence of the small size of sire and dams of the SNL pig population. Furthermore, linkage analyses, accomplished by using HAPLOVIEW software (version 4.2), showed a complete linkage disequilibrium among the three polymorphic sites with a D' = 1.0 and a $r^2 = 1.0$ for all two loci pairwise comparisons. As a consequence, the three loci segregate as a block giving rise to only two haplotypes (A = –366A ‒ –225G ‒ –182C and B = –366G ‒ –225C ‒ –182T; Table 2) and, therefore, three genotypes (A/A, A/B, and B/B). According to the comparison with data obtained by Aslan *et al*. (2012), our A and B haplotypes should correspond to their HAP2 and HAP1 haplotypes, respectively.

Meat quality

The results of this study showed that, in the SNL pig, the two observed *IGF2* haplotypes are associated with effects on both the increase in muscle mass and on meat quality characteristics ($P < 0.05$). In fact, B/B animals are characterised by a significantly higher weight of LL and P muscles respect to A/A animals (Fig. 1). The value observed in heterozygous individuals put forward the possibility of dominance effects at this locus. These results are supported by previous studies on the effect of the *IGF2* polymorphism on muscle mass whose variation is strongly affected by the different *IGF2* genotypes (15–30%) (Jeon *et al*. 1999; Nezer *et al*. 1999). The *IGF2* gene seems to have an autocrine/paracrine mode of action on proliferation and differentiation of multiple cell types within the muscle tissue, including intramuscular adipocytes (Florini *et al*. 1991; Oksbjerg *et al*. 2004). In addition, Clark (2015) suggested that the *IGF2* polymorphism could influence, in the prenatal phase, muscle fibres hyperplasia, which contributes to muscle mass increases. Finally, according to Kokta *et al*. (2004) the *IGF2* polymorphism could be responsible for changes in the distribution of nutrients in animal metabolism, resulting in the growth of lean tissue rather than adipose tissue. Consistent with these results, in the current trial B/B animals are characterised by a significantly lower subcutaneous fat depth respect to A/A animals (Table 3). The effect of the *IGF2* genotypes on chemical and rheological properties of the SNL meat is shown in Table 3. Overall, the A and B *IGF2* haplotypes are associated with significant differences in fat, protein, and haem iron values, whereas no significant associations were found for DM and ash content. In particular, B/B animals showed a meat with the highest protein content and the lowest haem iron content ($P < 0.05$), whereas IMF content was the highest in A/A animals ($P < 0.05$). The last finding is consistent with the results reported by other authors (Van den Maagdenberg *et al*. 2008; Aslan *et al*. 2012; Burgos

Table 1. Genotypes and allele frequencies at the three *IGF2* polymorphic sites in the Suino Nero Lucano pig population

*, $P < 0.005$

Locus	No. of pigs	Genotypes			Allele frequencies		χ^2 (d.f. = 1)
–366G > A	166	A/A 118	A/G 36	G/G 12	f_A 0.82	f_G 0.18	12.21*
–225C > G	166	G/G 118	G/C 36	C/C 12	f_G 0.82	f_C 0.18	12.21*
–182*T* > C	166	C/C 118	C/T 36	T/T 12	f_C 0.82	f_T 0.18	12.21*

et al. 2012; Oczkowicz *et al*. 2012) who correlated the *IGF2* polymorphism with effects on the IMF content. The IMF level strongly affects meat quality in terms of its nutritional value and sensory properties. In fact, Affentranger *et al*. (1996) established that ~2–2.5% of IMF is the minimum acceptable level, whereas Fernandez *et al*. (1999) reported that an excess of visible fatness could reduce consumer acceptability of fresh meat and cured products. Furthermore, Pugliese and Sirtori (2012) evidenced a more intense fat aroma in dry cured hams obtained from individuals with high levels of IMF as the high content in intramuscular triglycerides is responsible for a high content of aromatic compounds. The increase in IMF can improve significantly meat quality through an increase in tenderness (van Laack *et al*. 2001; Huff-Lonergan *et al*. 2002). van Laack *et al*. (2001) evidenced a significant linear negative correlation between IMF and WBS, in Duroc meat. Furthermore, it is known that tenderness is correlated with WHC (Joo *et al*. 2013). The WHC value is also correlated with the appearance of raw meat, its behaviour during cooking and the sensorial quality of the product. Our results showed that meat from A/A animals, characterised by a higher value of IMF, also showed the lowest WBS value compared with that from B/B animals ($P < 0.05$), and the lowest drip loss value respect to that of both A/B and B/B animals (Table 3; $P < 0.05$). Meat quality is closely linked to the fatty acid composition of IMF. The influence of the *IGF2* genotypes was observed on both monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) content, whereas no significant effect was found on saturated fatty acids (SFA) content (Fig. 2). Meat from B/B animals showed the highest PUFA percentage and the lowest MUFA percentage respect to that of A/A animals ($P < 0.05$). As SFA and MUFA are mainly located in triacylglycerols and PUFA in the cell membrane phospholipids, and as the content of muscle triglycerides is strongly correlated with fat content (De Smet *et al*. 2004),

Table 2. Expected and observed frequencies of the eight potential IGF2 haplotypes in the Suino Nero Lucano pig population

Haplotypes	Expected frequencies	Observed frequencies
–366A – –225G – –182C[A]	0.550	0.82
–366A – –225G – –182T	0.121	0.00
–366A – –225C – –182C	0.121	0.00
–366A – –225C – –182T	0.027	0.00
–366G – –225G – –182C	0.121	0.00
–366G – –225G – –182T	0.027	0.00
–366G – –225C – –182C	0.027	0.00
–366G – –225C – –182T[B]	0.006	0.18

[A]A haplotype.
[B]B haplotype.

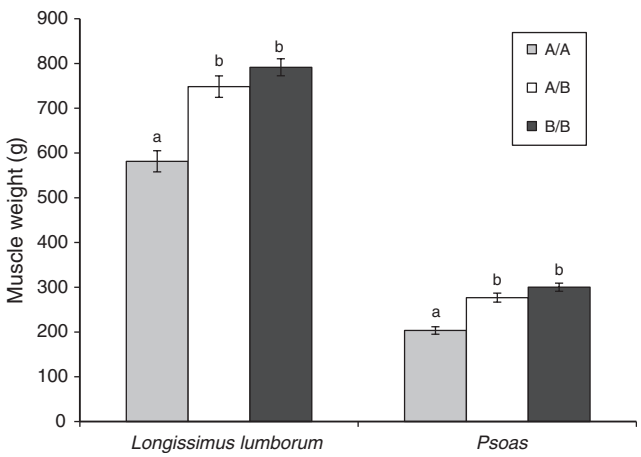

Fig. 1. Weight (g) of sample cut (between cranial end of the 2nd lumbar vertebra and the caudal end of the 5th lumbar vertebra) of *Longissimus lumborum* and *Psoas* muscles from Suino Nero Lucano pigs, clustered according to the *IGF2* genotype (A/A, A/B, and B/B; 10 individuals for each genotype, respectively), at the same carcass weight. Different letters within each muscle indicate significant differences ($P < 0.05$).

Table 3. Age at slaughter, carcass weight, subcutaneous fat depth, chemical composition and rheological properties of *Longissimus lumborum* muscles from Suino Nero Lucano pigs, clustered according to the *IGF2* genotype

Values are means ± s.e.m. (standard error of means). Means in the same row with a common letters following them are not significantly different ($P > 0.05$). DM, dry matter; WBS, Warner–Bratzler Shear

	Genotypes			s.e.m.	*P*-value
	A/A	A/B	B/B		
No. of pigs	10	10	10	–	–
Age at slaughter (day)	551a	542a	528a	4.84	0.082
Carcass weight[A] (kg)	111.8a	112.4a	117.4a	2.58	0.211
Subcutaneous fat depth[B] (cm)	4.7a	4.0b	3.8b	0.11	0.002
DM (g/100 g meat)	29.21a	28.61a	27.45a	0.62	0.146
Fat (% DM)	23.05a	23.32a	22.06b	0.19	<0.001
Protein (% DM)	73.04a	72.66a	73.96b	0.19	<0.001
Ash (% DM)	3.92a	4.02a	3.98a	0.08	0.539
Haem iron (ppm)	11.53a	10.31a	9.64b	0.36	0.003
Drip loss (% per 24 h)	2.74a	3.18b	3.68c	0.12	<0.001
WBS (N)	11.66a	12.52a,b	13.26b	0.32	0.005

[A]Including head and tail.
[B]Subcutaneuos fat depths between 2nd and 5th lumbar vertebrae.

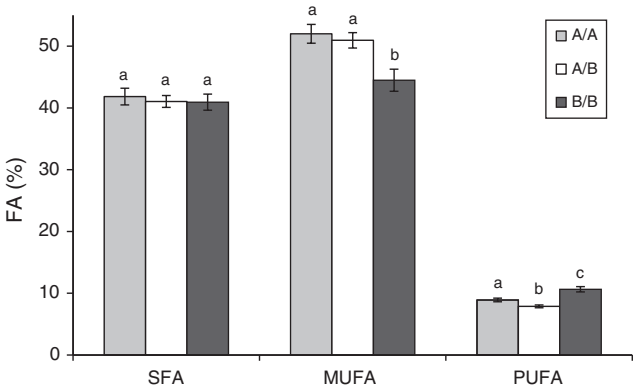

Fig. 2. Saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) content (%) in *Longissimus lumborum* muscle from Suino Nero Lucano pigs, clustered according to the *IGF2* genotype (A/A, A/B, and B/B; 10 individuals for each genotype, respectively). Different letters within each fatty acid (FA) class indicate significant differences ($P < 0.05$).

these results agree with the lower IMF value observed in meat from B/B animals. The observed variability in acid profile of IMF from SNL meat is consistent with the results reported by López-Buesa *et al*. (2014), who found that *IGF2* has strong additive effects on several carcass conformational traits and on fatty acid composition in several anatomical locations. On the contrary, Gardan *et al*. (2008) found no effect of *IGF2* polymorphism on muscle lipid content and fatty acid composition. The effect of *IGF2* genotypes on colourimetric parameters and myoglobin content of the LL muscles from SNL pigs is shown in Table 4. Meat from B/B animals showed the highest values of the three considered colourimetric parameters, even if only *L** value was statistically different from the one detected in meat of A/A animals (59.34 vs 49.07, respectively). The influence of *IGF2* polymorphism on *L** colourimetric parameter was also found by Burgos *et al*. (2012), in *Longissimus* muscle, and by Van den Maagdenberg *et al*. (2008) in *Triceps brachii* muscle. Meat colour is defined by the extent of myoglobin oxygenation and the oxidative status of the haem iron (Faustman *et al*. 2010), which is responsible for the characteristic red colour (Mancini and Hunt 2005). In particular, meat colour is largely related to the relative proportions of MbO_2 (bright red), Mb (dark red), and MetMb (grey-brown), which are in a dynamic cycle wherein all three forms are constantly converted and in equilibrium with each other. Overall, MbO_2 is the colour that consumers associate with freshness (Boles and Pegg 2001). In this study, LL muscles from A/A animals showed the highest percentage of MetMb and the lowest percentage of Mb and MbO_2 compared with those from A/B and B/B animals; whereas no significant difference was found between A/B and B/B animals (Fig. 3).

Table 4. Colourimetric characteristics and myoglobin forms (%) of *Longissimus lumborum* muscles from Suino Nero Lucano pigs, clustered according to the *IGF2* genotype

Values are means ± s.e.m. (standard error of means). Means in the same row with a common letter following them are not significantly different ($P > 0.05$)

	Genotypes			s.e.m.	*P*-value
	A/A	A/B	B/B		
No. of pigs	10	10	10	–	–
*L**	49.57a	57.73b	59.38b	1.29	<0.001
*a**	21.63a	21.84a	24.14a	0.46	0.083
*b**	18.29a	18.48a	18.85a	0.54	0.762

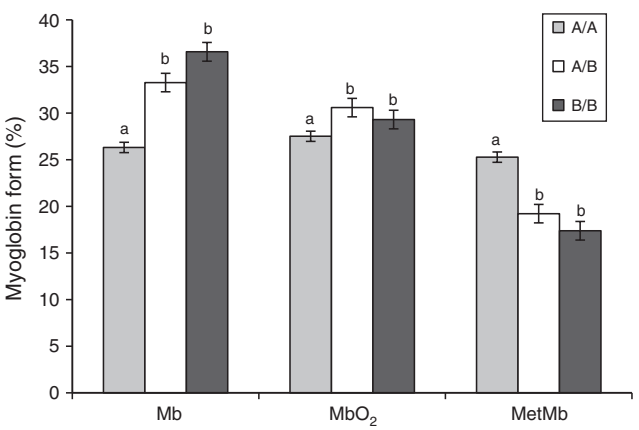

Fig. 3. Percentage of myoglobin (Mb), metmyoglobin (MetMb) and oxymyoglobin (MbO_2) ($P < 0.001$, $P < 0.048$, and $P < 0.001$, respectively) in *Longissimus lumborum* muscles from Suino Nero Lucano pigs, clustered according to the *IGF2* genotype (A/A, A/B, and B/B; 10 individuals for each genotype, respectively). Different letters within each myoglobin forms indicate significant differences ($P < 0.05$).

Conclusions

Results obtained in this study confirm that in an autochthonous population, characterised by a limited number of individuals, such as the Suino Nero Lucano pig, a certain level of variability in meat quality can be observed. In addition, for many of the considered traits, the polymorphism at the *IGF2 locus* is associated with strong effects on this variability. In particular, B/B animals, at the same carcass weight, showed a higher LL and *Psoas* weight and a lower subcutaneous fat depth, compared with A/A animals. Furthermore, meat from B/B animals showed higher protein content, *L** and shear-force values, and lower IMF content but with a higher PUFA percentage compared with meat from A/A ones. These findings confirm what was already suggested by other authors, who consider the *IGF2* gene as a QTL affecting meat quality and slaughter traits. The genetic analyses of the *IGF2 locus* evidenced that the conservation project of the SNL population must pay particular attention to the low level of genetic variability and high level of inbreeding. However, the presence of only two out of the eight possible haplotypes at the *IGF2 locus* could be useful to trace products of SNL origin. Furthermore, the presence in the population of two haplotypes associated with low and high levels of fat opens the possibility to select some SNL pigs for the production of fresh lean meat.

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