

## PAPER

## Investigation of a short interspersed nuclear element polymorphic site in the porcine *vertnin* gene: allele frequencies and association study with meat quality, carcass and production traits in Italian Large White pigs

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### Abstract

A 291 bp short interspersed nuclear element (SINE) insertion in the porcine *vertnin* (*VRTN*) gene on porcine chromosome 7 was shown to affect vertebral number and several production traits: allele Q (with the insertion) increases vertebral number compared to the wild type allele (WT, without insertion). In this study we genotyped this polymorphism in eight pig breeds (Italian Large White, Italian Duroc, Italian Landrace, *Cinta Senese*, *Mora Romagnola*, *Casertana*, *Apulo Calabrese*, and *Nero Siciliano*) and in Italian wild boars to evaluate allele frequency distribution of the two alleles. Allele Q was the most frequent in Italian Landrace and Italian Duroc (0.738 and 0.545, respectively) whereas it was the less frequent in all other breeds and was absent in wild boars. Association study was carried out in two Italian Large White samples. These two groups of animals were constituted by performance tested pigs for which estimated breeding values (EBV) and random residuals (RR) for several traits (average daily gain, back fat thickness, feed:gain ratio, lean cuts and ham weight) were calculated: i) 270 pigs chosen without any criteria (random group),

that were also measured for several meat quality traits; ii) 560 gilts with extreme and divergent EBV for back fat thickness. For these animals vertebral number was not available. Results of the association analyses indicated that allele Q was associated with a lower ham weight, confirming indirectly, the negative correlation reported by other studies between vertebral number and this trait. No other trait was associated with the analysed *VRTN* polymorphism.

### Introduction

A large number of QTL studies for production and morphological traits have been carried out in pigs (Hu *et al.*, 2013). However, responsible genes have been identified for few QTL only. In particular, an important QTL for lean meat/fat deposition identified in the p arm of porcine chromosome (SCC) 2 was shown to be due to a single nucleotide polymorphism (SNP) in intron 3 of the insulin like growth factor 2 (*IGF2*) gene (*intron3-g.3072G>A*) (Van Laere *et al.*, 2003). We recently investigated the effects of this *IGF2* polymorphism in Italian Large White pigs and reported a strong significant association with lean meat content, back fat thickness, average daily gain and feed gain conversion rate (Fontanesi *et al.*, 2010, 2011, 2012). Other studies identified QTL for vertebral number on SSC1 and SSC7 (Wada *et al.*, 2000; Sato *et al.*, 2003; Mikawa *et al.*, 2005). Mikawa *et al.* (2007) showed that a missense mutation in the nuclear receptor subfamily 6, group A, member 1 (*NR6A1*) gene is the most probable causative mutation for the QTL on SSC1. This mutation is fixed for the allele associated with larger number of thoracic and lumbar vertebrae in most commercial pig breeds (Mikawa *et al.*, 2007; Yang *et al.*, 2009; Ruben *et al.*, 2012), including Italian Large White (Fontanesi *et al.*, manuscript in preparation). Therefore, this polymorphism is not suitable for association studies with production traits in these breeds. Subsequently, Mikawa *et al.* (2011) fine mapped the QTL on SSC7 overlapping or residing close to QTL regions for body composition (Sato *et al.*, 2003, Mikawa *et al.*, 2005), growth rate (Nezer *et al.*, 2002; Edwards *et al.*, 2008) and fat deposition (Malek *et al.*, 2001; Liu *et al.*, 2007), and identified the underlying gene associated with variation in vertebral number. This gene, named *vertnin* (*VRTN*), had never been characterized before in any species, even if orthologs appear to exist in mammals, birds

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and fishes (Mikawa *et al.*, 2011). According to this study, an insertion of 291 bp of a PRE1 repeated sequence (one of the porcine short interspersed nuclear element classes; SINE) into intron 1 of the *VRTN* gene together or in alternative with nine SNPs in the promoter region of this gene (in linkage disequilibrium with the SINE insertion in the analysed population) might be the responsible mutations of the favourable QTL allele on vertebral number (allele Q) compared to the wild type (WT) allele (without insertion), that might reduce vertebral number. Results of Mikawa *et al.* (2011) were recently confirmed and refined by Fan *et al.* (2013) who suggested that the most probable causative mutations of the SSC7 QTL is the SINE insertion in intron 1 or/and an SNP in the promoter region, both being in linkage disequilibrium. As vertebral number is correlated with body length that, in turn, is correlated with other productive and reproductive traits (*e.g.*, Borchers *et al.*, 2004), it is important to evaluate the effect of variability in the *VRTN* gene with meat quality and carcass traits. Hirose *et al.* (2013) identified association between this gene and body length, body weight, chest circumference, cannon circumference of forelegs, eye muscle area, and intramuscular fat percentage in a Duroc population.

In this study we genotyped the 291 bp SINE

insertion of the *VRTN* gene i) in different pig breeds to evaluate frequency distribution of the two alleles and ii) in two Italian Large White pig groups to evaluate association between this polymorphic site and back fat thickness, lean meat content, ham weight, growth rate and efficiency and several meat quality traits.

## Materials and methods

### Animals and traits

Triplets of Italian Large White full sibs pigs (two females and one castrated male) are currently performance tested at the Central Test Station of the National Pig Breeder Association (ANAS) for the genetic evaluation of a boar from the same litter (sib-testing). The evaluation period starts when pigs are 30-45 days old and it ends when animals reach 155±5 kg live weight. The nutritive level is *quasi ad libitum*, meaning that about 60% of the pigs are able to ingest the entire supplied ration. Daily feed intake and bimonthly body weight measures are collected and average daily gain (ADG) and feed:gain ratio (FGR) calculated. At the end of test, animals are transported to a commercial abattoir where they are slaughtered. After slaughtering, back fat thickness (BFT) on the carcasses at the level of *Musculus gluteus medius*, lean cuts weight (LC; necks and loins) and ham weight (HW) are measured.

Two groups of Italian Large White pigs were used in association analyses to have an independent confirmation of the association results for the common traits available for both populations. The first group was constituted by 270 animals (180 females and 90 castrated males from 59 sires and 153 different triplets) slaughtered on six different dates in the same abattoir. These pigs were not selected by any criteria (random group). Phenotypes recorded for these animals were those routinely measured as described above and for which estimated breeding values (EBVs) and random residuals (RRs) were calculated (see below for details about EBV and RR calculation). Besides, other meat quality indicators were recorded on *Musculus semi-membranosus* as already reported (Fontanesi *et al.*, 2008; Russo *et al.*, 2008): i) pH<sub>1</sub> (at 2 h *post-mortem*); ii) pH<sub>u</sub> (at 24 h *post-mortem*) iii) glycolytic potential, calculated as the sum of: 2[glycogen + glucose + glucose-6-phosphate] + [lactate] (Monin and Sellier, 1985); iv) cathepsin B activity at 24 h *post-*

*mortem*. The second group of Italian Large White pigs was constituted by two extreme and divergent groups of females (one female per triplet), performance tested in the period 1996-2007. Pigs were chosen according to their estimated breeding value (EBV) for BFT (280 pigs with most negative and 280 with most positive EBV) within a performance tested population of ~12,000 pigs (details of EBV calculation are reported below). All sampled pigs were at least two-generation unrelated (Fontanesi *et al.*, 2012; 2013b). Average BFT EBV in the negative and positive selected groups of pigs were -9.4±1.6 mm and +8.0±5.95 mm, respectively. For these animals not only BFT EBV was available but also ADG EBV, FGR EBV, LC EBV and HW EBV and their corresponding RRs (see below).

A total of 170 animals belonging to 8 different breeds/subspecies (Italian Duroc, n=22; Italian Landrace, n=21; *Cinta Senese*, n=21; *Mora Romagnola*, n=22; *Casertana*, n=22; *Apulo Calabrese*, n=20; *Nero Siciliano*, n=20; Italian wild boar sampled in Emilia Romagna region, n=22), for which no phenotypic traits were available, were used to obtain first information about allele frequency distribution.

### Genotyping and sequencing

Genomic DNA was extracted from liophilized muscle samples (for the 270 Italian Large White pigs), from liophilized blood (for the 560 Italian Large White pigs), from whole blood, skin tissues or hair roots (for the pigs used for allele frequency evaluation) using the Wizard<sup>®</sup> Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA) (for the whole blood and skin tissues) or a standard phenol-chloroform protocol (for the other tissues). PCR primers used for the amplification of the AB554652:g.20311\_20312ins291 SINE insertion were as reported in Mikawa *et al.* (2011): 5'-ggcaggaaggt-gttgtta-3' (forward) and 5'-gactggcctctgcttg-3' (reverse). PCR was carried out using a 2720 thermal cycler (Life Technologies, Carlsbad, CA, USA) in a 20 L reaction volume containing ~50 ng genomic DNA, 1 U DNA EuroTaq DNA polymerase (EuroClone Ltd., Paington, Devon, UK), 1X Euro Taq PCR buffer, 2.5 mM dNTPs, 10 pmol of each primer and 1.5 mM of MgCl<sub>2</sub>. PCR profile was as follows: 5 min at 95°C; 35 amplification cycles of 30 s at 95°C, 30 s at 56°C, 30 s at 72°C; 5 min at 72°C. Amplified fragments were electrophoresed on 1.5% agarose gels and visualized with 1X GelRed Nucleic Acid Gel Stain (Biotium Inc., Hayward, CA, USA). Allele of 411 bp (with the 291 bp insertion) was asso-

ciated with the Q allele (favourable allele for increased vertebral number) whereas the allele of 120 bp (without insertion) is considered the Wild Type (WT) allele (Mikawa *et al.*, 2011; Fan *et al.*, 2013). Amplified fragments from 3 pigs each of two homozygous genotypes were sequenced to confirm the correspondence of the expected fragments with the obtained amplicons. Sequencing and sequence analysis were carried out as previously described (Fontanesi *et al.*, 2010).

### Statistical analyses

Estimated Breeding Values in the Italian Large White pigs were calculated using a BLUP-Multiple Trait-Animal Model with different models for each trait. Depending on the trait, models included the fixed effects of sex, batch on trial, inbreeding coefficient of the animal, interaction of sex by age at slaughtering, date of slaughtering and the random effects of litter and animal. Random residuals were calculated for the considered EBVs. RRs were obtained by using linear fixed models including the same factors used for each trait in the BLUP-Multiple Trait Animal Model (Fontanesi *et al.*, 2010). In the first group of Italian Large White pigs (n=270) association analysis between the g.20311\_20312ins291 insertion and EBVs or RRs were assessed by using the general linear model (GLM) procedure of SAS, release 9.2 (SAS Inst. Inc. Cary, NC, USA). The models included only the fixed effects of individual marker genotypes. All other factors contributing to variability of the investigated traits were already considered in the calculation of EBV or RR. For meat quality traits determined in this group of investigated Italian Large White pigs, the procedure MIXED of SAS version 9.2 was applied to a model that included date of slaughtering, sex and genotype. For the group of pigs chosen for selective genotyping based on extreme values of EBVs for BFT (n=560), Chi square test of differences in allele and genotype frequencies between the positive and negative groups were used (for this group of pigs a few animals were not genotyped). For all other traits available on these animals (ADG, FGR, HW, and LC) association analyses were carried out by using both EBVs and RRs with the procedure GLM of SAS in a model that included the tail as fixed effect and the *VRTN* genotypes as nested effects within tail. Correlations between BFT EBV and all other traits in this group of pigs were reported in Fontanesi *et al.* (2013a).

## Results and discussion

Table 1 reports information about allele frequencies of the g.20311\_20312ins291 *VRTN* polymorphism in several Italian pig breeds and in Italian wild boars. Allele Q was the most frequent (0.738) in Italian Landrace, a breed that is known to have a long carcass, and Italian Duroc (0.545). Almost the same frequency was observed in a Chinese Landrace population (0.71) and in a Chinese Duroc population (0.54), respectively (Fan *et al.*, 2013). Hirose *et al.* (2013) reported a fre-

quency of of 0.60 for allele Q in a Japanese Duroc population. This allele was the less frequent in all other investigated domestic breeds (Table 1). Interestingly, its frequency in Italian Large White (0.357) was opposite from what reported in Chinese Large White pigs (0.66; Fan *et al.*, 2013), suggesting different selection pressures at this locus in different Large White populations. Among the local Italian breeds, *Mora Romagnola*, that was crossed in the past with Duroc blood, had the highest frequency of allele Q (0.477), followed by *Casertana* (0.364), *Apulo Calabrese* (0.125), *Cinta Senese* (0.119) and *Nero*

*Siciliano* (0.050). As expected, sampled Italian wild boars carried only the WT allele.

Association between the *VRTN* polymorphism and several production traits was carried out in two Italian Large White populations. Results of the association study in the random group of Italian Large White pigs (Table 2) showed a suggestive significant effect only on HW EBV and HW RR (P=0.054 and P=0.071, respectively). Animals with Q/Q genotype had a lower HW than pigs with WT/WT genotype (P<0.05). None of the other traits resulted associated with the *VRTN* polymorphism in this group of pigs (Table 2).

**Table 1. Genotype and allele frequencies of the *VRTN* g.20311\_20312ins291 polymorphism in different pig breeds and in Italian wild boars.**

Breeds/subspecies	N of genotyped animals	Genotypes – n of pigs			Allele frequencies	
		Q/Q	Q/WT	WT/WT	Q°	WT°
Italian Large White <sup>†</sup>	824	132	324	368	0.357	0.643
Italian Duroc	22	7	10	5	0.545	0.455
Italian Landrace	21	13	5	3	0.738	0.262
<i>Cinta Senese</i>	21	2	1	18	0.119	0.881
<i>Mora Romagnola</i>	22	5	11	6	0.477	0.523
<i>Casertana</i>	22	4	8	10	0.364	0.636
<i>Nero Siciliano</i>	20	0	2	18	0.050	0.950
<i>Apulo Calabrese</i>	20	0	5	15	0.125	0.875
Italian Wild Boar	22	0	0	22	0.000	1.000

°Q and WT alleles are as defined in Materials and methods; <sup>†</sup>pigs used in the association studies.

**Table 2. Association between the *VRTN* g.20311\_20312ins291 polymorphism (Q and WT alleles) and several meat quality and production traits in the 270 Italian Large White pigs. Least square means±standard errors of the different genotypes are presented.**

Traits	Genotypes			P
	Q/Q n=44	Q/WT n=85	WT/WT n=141	
EBV ADG, g	32.364 ± 4.025	33.106±2.896	33.901±2.248	0.939
EBV LC, kg	2.293±0.279	1.938±0.200	1.949±0.156	0.520
EBV BFT, mm	-2.475±0.573	-1.694±0.412	-2.184±0.320	0.483
EBV HW, kg	0.409±0.093	0.553±0.066	0.656±0.050	0.054
EBV FGR	-0.135±0.023	-0.148±0.017	-0.153±0.013	0.792
RR ADG, g	-1.588±11.947	-7.547±8.497	1.541±6.597	0.700
RR LC, kg	0.390±0.409	-0.136±0.291	-0.042±0.226	0.559
RR BFT, mm	-0.638±0.772	0.181±0.549	-0.073±0.426	0.689
RR HW, kg	-0.293±0.199	-0.125±0.140	0.163±0.106	0.071°
RR FGR	0.014±0.049	0.014±0.035	0.025±0.027	0.960
pH <sub>1</sub>	5.942±0.0370	5.923±0.027	5.922±0.022	0.882
pH <sub>24</sub>	5.624±0.031	5.655±0.023	5.665±0.019	0.485
Glycogen, µmol	50.315±3.505	48.057±2.633	48.347±2.193	0.843
Lactate, µmol	52.880±2.344	58.108±1.729	57.214±1.359	0.170
GP, µmol	103.100±3.545	106.270±2.654	105.490±2.188	0.739
Catb, nmol	1.159±0.022	1.185±0.027	1.140±0.022	0.368

EBV, Estimated Breeding Value; ADG, average daily gain; LC, lean cuts; BFT, back fat thickness; HW, ham weight; FGR, feed:gain ratio; RR, Random Residuals; pH<sub>1</sub>, pH at 2 h *post-mortem*; pH<sub>24</sub>, pH at 24 h *post-mortem*; glycogen and lactate (µmol of lactic acid equivalent per g of fresh muscle); GP, glycolytic potential (µmol of lactic acid equivalent per g of fresh muscle); Catb, cathepsin B activity (nmol of 7-amino-4-methylcoumarin released/min/g muscle). °P<0.10.

Table 3 shows *VRTN* genotype and allele distributions in the two extreme and divergent groups of pigs for BFT EBV. No significant differences were observed between the two tails, confirming what was observed in the random group of Italian Large White pigs, namely *VRTN* is not associated with BFT in this breed. Association analyses for the other traits in this second population of Italian Large White pigs showed that the genotyped marker affects HW EBV and HW RR ( $P < 0.01$ ), confirming the trend observed in the random group of pigs, with the same direction of the effect of the two alleles (namely, allele Q decreases HW). In this population, significant association ( $P < 0.05$ ) was observed also with FGR RR. This result was not confirmed using EBVs for the same trait and, for this reason, it should be further validated.

For all animals used in the association analyses, vertebral number was not available. However, weight of lean cuts recorded in the investigated pigs, determined by the weight of two necks and two loins, might contain indirectly information on the number of vertebrae of the pigs. Necks contain cervical and thoracic vertebrae whereas loin cuts include lumbar, sacral and caudal vertebrae. Total number of vertebrae was correlated with total

number of vertebrae, rib number, body length and body weight (Borchers et al., 2004; Ren et al., 2012). The QTL on SSC7 centred on *VRTN* affects mainly thoracic and total number of vertebrae (Mikawa et al., 2005; Ren et al., 2012). According to these studies, the lack of association between *VRTN* and LC we observed may be explained by the fact that there might be a negative or poor correlation between the length of lean cuts and their weight as, usually, shorter loins have a larger *Longissimus dorsi* area (Buttazzoni et al., unpublished results). However this putative relationship should be further investigated. A negative correlation between presacral vertebrae number and HW was already reported by other studies (Tohara et al., 1965; Borchers et al., 2004). This indirectly means that the *VRTN* Q allele would be associated with a reduced HW, as we observed in our association studies (Tables 2 and 4). Selection towards increased HW or to maintaining an appropriate HW for dry-cured ham production would have contributed to a lower frequency of allele Q in the Italian Large White breed. Hirose et al. (2013) evaluated the effect of the *VRTN* polymorphism on several production traits measured in a Duroc population and did not evidence any significant results

on performance and carcass traits, i.e. ADG and BFT, similarly to what we observed in Italian Large White pigs. These results could indicate that *VRTN* does not have important negative pleiotropic effects on other production traits that constitute the selection index of this breed.

## Conclusions

This is the first investigation on Italian pig breeds concerning the *VRTN* gene, that in other studies was shown to affect vertebral number and correlated traits. Association analyses were carried out in two Italian Large White groups of pigs on several production and carcass traits using EBV and RR. Significant effects of the analysed polymorphic site were observed only for HW: allele Q may decrease HW whereas allele WT may increase this trait value. All other traits were not significantly convincingly associated with this gene. However, vertebral number was not available in the genotyped animals and no recent study has reported such information in any Italian heavy pig breeds. As *VRTN* might be a causative gene, it will be interesting to

**Table 3. Distribution of *VRTN* genotypes and alleles in the two groups of Italian Large White pigs with extreme and divergent (negative and positive tails) estimated breeding values for back fat thickness. Chi square tests compare genotype and allele distributions in the two tails.**

	Genotypes, n of animals (frequency)			Alleles, n (frequency)	
	Q/Q	Q/WT	WT/WT	Q°	WT°
BFT EBV tails/test					
Negative	38 (0.138)	118 (0.429)	119 (0.433)	194 (0.353)	356 (0.647)
Positive	50 (0.179)	121 (0.434)	108 (0.387)	221 (0.396)	337 (0.604)
Chi square (P)	0.337			0.136	

BFT, back fat thickness; EBV, Estimated Breeding Value. °Q and WT alleles are as defined in Materials and methods.

**Table 4. Probability, least square means±standard errors obtained in the association study by general linear model procedure between the *VRTN* polymorphism and estimated breeding values, and random residuals for average daily gain, feed gain ratio, ham weight and lean cuts in the tails of pigs with extreme and divergent estimated breeding values for back fat thickness.**

Tail°	Genotype (n of pigs)	ADG-EBV	FGR-EBV	HW-EBV	LC-EBV	ADG-RR	FGR-RR	HW-RR	LC-RR
Negative	Q/Q (38)	37.6329±4.618	-0.1445±0.0276	0.489±0.093	4.532±0.245	-0.021±0.013	0.077±0.057	0.235±0.192	1.989±0.442
	Q/WT (118)	38.1448±2.621	-0.196±0.016	0.619±0.053	4.299±0.139	-0.006±0.007	-0.054±0.032	0.113±0.105	1.578±0.242
	WT/WT (119)	40.6813±2.609	-0.196±0.0156	0.798±0.053	4.309±0.138	0.010±0.007	-0.110±0.0301	0.638±0.102	1.801±0.236
Positive	Q/Q (50)	6.820±4.0259	-0.008±0.024	0.043±0.081	-1.329±0.214	-0.002±0.010	0.047±0.046	-0.495±0.105	-1.741±0.359
	Q/WT (121)	6.587±2.588	-0.037±0.015	0.108±0.052	-1.449±0.137	-0.004±0.007	0.089±0.030	-0.342±0.101	-1.762±0.234
	WT/WT (108)	8.602±2.739	-0.051±0.0164	0.220±0.055	-1.409±0.145	0.005±0.007	0.037±0.033	-0.314±0.109	-1.596±0.251
P		0.923	0.275	0.006 <sup>‡</sup>	0.914	0.170	0.043 <sup>‡</sup>	0.007 <sup>‡</sup>	0.897

ADG, average daily gain; EBV, estimated breeding values; FGR, feed gain ratio; HW, ham weight; LC, lean cuts; RR, random residuals. °Data are reported for each genotype within tail (tails are constituted by pigs with extreme and divergent back fat thickness EBV); <sup>‡</sup> significant results.

further evaluate its association with number of vertebrae of the different body regions and, eventually, their correlation with other production traits in Italian Large White as well as in other Italian heavy pig breeds.

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