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Allelic frequencies of NR6A1 and VRTN, two genes that affect vertebrae number in diverse pig breeds: A study of the effects of the VRTN insertion on phenotypic traits of a Duroc × Landrace–Large White cross

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ABSTRACT

A SNP (748 C > T) in the NR6A1 gene and an insertion (g.20311_20312ins291) in the VRTN gene have been shown to affect vertebrae number in the pig. The allelic frequencies of both genes were investigated in six western breeds and the effects of the VRTN insertion on some phenotypic traits in a Duroc × Landrace/Large White cross. The NR6A1 c. 748T allele, associated with higher number of vertebrae, appeared to be fixed in most studied breeds except in Iberians. The VRTN insertion (Ins allele) shows ample variability in all studied breeds although the allelic frequency of Ins seems to be larger in breeds with a greater history of genetic selection. Ins is associated with an increase in weight at slaughter, in loin and rib primal cut proportions, and with modified meat quality properties such as cooking loss, intramuscular fat content or yield after curing. We discuss the usefulness of both gene markers for pig selection.

1. Introduction

The number of vertebrae in pigs varies considerably. It is an important trait in pig production because it directly determines the size of important meat cuts such as the loin and also has an overall effect on carcass conformation. One extra vertebra expands the carcass length up to 80 mm (King & Roberts, 1960). While wild boars and indigenous breeds have 19 vertebrae, western commercial breeds have a few more ($n = 21$–23) (King & Roberts, 1960) as a result of long-term selection for enlarged size.

Two quantitative trait loci (QTL) for vertebral number have been identified in pigs: one on chromosome 1 (SSC1) (Mikawa et al., 2005; Wada et al., 2000) and the other on chromosome 7 (SSC7) (Mikawa et al., 2005). The most likely causative mutation underlying the QTL on SSC1 is a base substitution in an orphan nuclear receptor, NR6A1 c. 748 C > T of GenBank sequence AB248749, which results in a proline to leucine substitution at codon 192 (Mikawa et al., 2007). Vertnin (VRTN), also known as "vertebrae development associated" gene (www.ncbi.nlm.nih.gov/gene/55237), was proposed as a strong candidate for the gene underlying the QTL reported on SSC7 (Mikawa et al., 2011). Recently, Fan et al. (2013) have identified the most likely causal variant of this QTL; it is an insertion of one fragment of 291 bp in the promoter region of the VRTN gene, g.20311_20312ins291, located in one transcription factor-binding site. This insertion alters the normal expression of the VRTN gene leading to the VRTN effect on vertebrae number. The allele with the insertion is denominated "Ins" in the literature whereas the wild type allele is denominated "Wt" or just "–". The effect of each of these mutations on vertebrae number is an increase of 0.55 to 0.6 thoracic vertebrae per allele (Mikawa et al., 2007, 2011).

While the allele of NR6A1 associated with increased vertebral number, allele T, seems to be fixed in all western breeds analyzed (Yang, Ren, Zhang, & Huang, 2009), genetic variation of VRTN has been detected in some European commercial breeds, but it has not been investigated in all such breeds, including the Pietrian or Iberian.

VRTN has several effects on other important traits besides vertebrae number. However, reports of its effects on other important traits are conflicting. For example, while Hirose et al. (2013) found that pigs with the Wt/Wt genotype had more intramuscular fat in the loin than those with the Ins/Ins genotype, Mikawa and Awata (2012) reported the opposite. Conflicting results have also been described on the influence of this gene on loin muscle area; Mikawa and Awata (2012) observed a smaller loin area associated with the Ins allele, but Hirose et al. (2013) didn't detect any statistically significant differences between the three genotypes for any loin traits. The effects of the vertnin

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gene on meat quality traits have only been evaluated by Mikawa and Awata (2012) who found that cooking losses were smaller in pigs with the Ins/Ins genotype than in Wt/Ins or Ins/Ins pigs.

In this work we analyzed the allelic frequencies of the NR6A1 c. 748 C > T and VRTN g.20311_20312ins291 mutations in six diverse western breeds, including breeds not yet studied for these mutations (Pietrain, Iberian, Porco Celta and Bízaro pigs). We also present a new RT-PCR method for the analysis of the NR6A1 c. 748 C > T single nucleotide polymorphism (SNP). We study the effects of VRTN alleles on several meat and carcass traits for which there exists some controversy in the literature and we discuss the usefulness of both gene markers for pig selection.

2. Material and methods

2.1. DNA extraction

DNA was extracted from diverse sources: from tail tissue of the Duroc × Landrace/Large White (Du × LD/LW) animals, from frozen muscle samples of Iberian pigs of two different companies, from semen from Pietrain pigs of Agropecuaria OBANOS SA (Marcilla, Spain), from frozen muscle samples of Porco Celta and hair samples of Bízaro pigs supplied by Dr. Carballo García (Universidad de Vigo, Spain), and from frozen muscles samples of wild boars, a gift of several hunting friends of our department. The number of analyzed animals of each breed or cross is indicated in Table 1. The extraction was performed using the GenomicPrepTM DNA isolation kit from Amersham Biosciences (Little Chalfont, UK) following the manufacturer's instructions as described in Burgos et al. (2006). DNA concentration in the extract was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific, Wilmington, DE, USA).

2.2. RT-PCR reaction for NR6A1 genotyping

This analysis of the SNP of the NR6A1 gene was performed by real time PCR using a DNA fragment of 360 bp of the gene previously amplified as described by Yang et al. (2009) and purified with the NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel, Düren, Germany). Primer and probe design were carried out with the Assay by Design method of Applied Biosystems. The PCR reactions were performed in an ABI-PRISM 7000 apparatus (Amersham Biosciences, USA). Each assay contained 6.3 μl of TaqMan Universal PCR Master Mix, 0.3 μl of 40X Assay Mix, 2.5 μl of DNA (2 ng/μl of the 360 bp amplicon) and double distilled H_2O to 12.5 μl. PCR was carried out using an initial cycle of 10 min at 95 °C followed by 50 cycles of 15 s at 92 °C and 1 min at 60 °C. Analysis of the results was carried out with the ABI-PRISM 7000 Sequence Detection System Software.

The primers used were SusNR6A1-F (GGGCTTCAGAGAGCAACCA) and SusNR6A1-R (GCCATGAATCCATTTAGTTCCACAGA), which amplified a 76-bp fragment containing the SNP. The probes were SusNR6A1-V, attached to VIC fluorescent label (CCTCAC*T*GGGCTCC) and SusNR6A1-M, attached to FAM fluorescent label (CTCAC*C*GGGC TCC). An internal standard dye, ROX, is incorporated in the reaction mix but it does not participate in the PCR reaction, serving as a control for pipetting errors and hardware or software problems. In each RT-PCR assay, two blanks with no added pig DNA but all the other mix components were included.

Table 1
Genotypic and allelic frequencies of NR6A1 c. 748 C > T in several pig populations.

	Genotypic frequency			Allelic frequency	
	TT	CT	CC	T	C
Wild boar ($n = 9$)	0.000	0.000	1.000	0.000	1.000
Iberian ($n = 34$)	0.617	0.265	0.118	0.750	0.250
Bízaro ($n = 12$)	1.000	0.000	0.000	1.000	0.000
Porco Celta ($n = 12$)	1.000	0.000	0.000	1.000	0.000
Pietrain ($n = 39$)	1.000	0.000	0.000	1.000	0.000
Du × LW/LD ($n = 20$)	1.000	0.000	0.000	1.000	0.000

2.3. Analysis of the VRTN g.20311_20312ins291genotype

This determination was carried out by the method of Fan et al. (2013). This is a procedure based on the analysis of the length of the PCR product obtained with a pair of primers designed to amplify a region of the VRTN gene that includes the point where this insertion is located.

2.4. Linkage disequilibrium analysis

A maximum likelihood of gametic frequencies and linkage disequilibrium between NR6A1 and VRTN genes in the Iberian population has been obtained from a genotypic count (Weir, 1996).

2.5. Animal material for composition analysis

Two hundred and two pigs of both sexes (the males were castrated) from 9 unrelated Duroc (Du) sires mated to 32 unrelated Landrace/Large White (LD/LW) dams were raised in an experimental farm in four different batches grown consecutively, fed ad libitum a standard diet and slaughtered at 114.5 +/− 18.9 kg, following the specifications of the "DO, Jamón de Teruel" (BOE, 2011).

2.6. Carcass processing

The pigs were stunned with CO_2, slaughtered early in the morning and chilled for 6–8 h. Backfat thickness was measured at the fifth vertebrae in the lumbar region (L_5) level using a caliper. The right side of the carcass was then divided into 10 meat cuts: ham, tenderloin, loin, shoulder, blade, belly, lard, ribs, dewlap, and leftover pieces as described in Burgos et al. (2012). All these cuts were immediately weighed.

2.7. Meat quality parameters

"Lab" color coordinates were measured with a Minolta Chromameter CR-200B equipped with a D65 illuminant and calibrated following manufacturer instructions. Loins were cut at approximately the third lumbar vertebrae (L_3) level and 6 measurements covering most of the loin area were immediately performed. Color was measured immediately after cutting the loins allowing no blooming to occur. Triplicate measurements of pH_{45} and pH_{24} were recorded in the *Longissimus lumborum* at approximately the second lumbar vertebrae (L_2) level using a PC300 Oakton Instrument equipped with a penetration Hamilton electrode (Bonaduz, Switzerland) calibrated using standard calibration protocols (i.e., against buffer of pH 4.01 and 7.00) at working temperatures (4 °C for pH_{24} and 37 °C for pH_{45} measurements). Eight loin slices from about the second lumbar to the twelfth thoracic vertebrae (L_2–T_{12}) were cut for water holding capacity (4) and cooking loss (4) determinations, which were performed as described by Honikel (1998). We report average values of all these measurements.

2.8. Chemical analysis

Muscle samples without any associated adipose tissue (*Biceps femoris*, *Psoas major*, *Longissimus lumborum*) were homogenized with a household mincer. Protein, ash and moisture content were determined using standard AOAC (1990) procedures. Lipids from 10 g of the homogenates were extracted by the method of Bligh and Dyer (1959) as modified by Hanson and Olley (1963). Fat content was analyzed gravimetrically from an aliquot of 5 ml of the chloroformic phase.