The insulin-like growth factor 2 (IGF2) gene intron3-g.3072G>A polymorphism is not the only Sus scrofa chromosome 2p mutation affecting meat production and carcass traits in pigs: Evidence from the effects of a cathepsin D (CTSD) gene polymorphism

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The insulin-like growth factor 2 (*IGF2*) gene intron3-g.3072G>A polymorphism is not the only *Sus scrofa* chromosome 2p mutation affecting meat production and carcass traits in pigs: Evidence from the effects of a cathepsin D (*CTSD*) gene polymorphism[1]

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ABSTRACT: The objective of this study was to evaluate the effects of mutations in 2 genes [*IGF2* and cathepsin D (*CTSD*)] that map on the telomeric end of the p arm of SSC2. In this region, an imprinted QTL affecting muscle mass and fat deposition was reported, and the *IGF2* intron3-g.3072G>A substitution was identified as the causative mutation. In the same chromosome region, we assigned, by linkage mapping, the *CTSD* gene, a lysosomal proteinase, for which we previously identified an SNP in the 3′-untranslated region (AM933484, g.70G>A). We have already shown strong effects of this *CTSD* mutation on several production traits in Italian Large White pigs, suggesting a possible independent role of this marker in fatness and meat deposition in pigs. To evaluate this hypothesis, after having refined the map position of the *CTSD* gene by radiation hybrid mapping, we analyzed the *IGF2* and the *CTSD* polymorphisms in 270 Italian Large White and 311 Italian Duroc pigs, for which EBV and random residuals from fixed models were calculated for several traits. Different association analyses were carried out to distinguish the effects of the 2 close markers. In the Italian Large White pigs, the results for *IGF2* were highly significant for all traits when using either EBV or random residuals (e.g., using EBV: lean cuts, $P = 2.2 \times 10^{-18}$; ADG, $P = 2.6 \times 10^{-16}$; backfat thickness, $P = 2.2 \times 10^{-9}$; feed:gain ratio, $P = 2.3 \times 10^{-9}$; ham weight, $P = 1.5 \times 10^{-6}$). No effect was observed for meat quality traits. The *IGF2* intron3-g.3072G>A mutation did not show any association in the Italian Duroc pigs, probably because of the small variability at this polymorphic site for this breed. However, a significant association was evident for the *CTSD* marker ($P < 0.001$) with EBV of all carcass and production traits in Italian Duroc pigs (lean content, ADG, backfat thickness, feed:gain ratio) after excluding possible confounding effects of the *IGF2* mutation. The effects of the *CTSD* g.70G>A mutation were also confirmed in a subset of Italian Large White animals carrying the homozygous genotype *IGF2* intron3-g.3072GG, and by haplotype analysis between the markers of the 2 considered genes in the complete data set. Overall, these results indicate that the *IGF2* intron3-g.3072G>A mutation is not the only polymorphism affecting fatness and muscle deposition on SSC2p. Therefore, the *CTSD* g.70G>A polymorphism could be used to increase selection efficiency in marker-assisted selection programs that already use the *IGF2* mutation. However, for practical applications, because the *CTSD* gene should not be imprinted (we obtained this information from expression analysis in adult skeletal muscle), the different modes of inheritance of the 2 genes have to be considered.

Key words: association study, cathepsin D gene, insulin-like growth factor 2 gene, Italian heavy pig, production trait, quantitative trait locus

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Table 1. Allele frequencies at the *IGF2* intron3-g.3072G>A and cathepsin D (*CTSD*) g.70G>A polymorphisms in different pig breeds

	IGF2			*CTSD*		
Breed[1]	No. of pigs	g.3072G	g.3072A	No. of pigs	g.70G	g.70A
Italian Large White	270	0.419	0.581	270	0.081	0.919
Italian Duroc	297	0.025	0.975	297	0.077	0.923
Italian Landrace	29	0.914	0.086	23	0.065	0.935
Cinta Senese	20	0.975	0.025	20	0.025	0.975
Casertana	17	1.000	0.000	17	0.000	1.000
Nero Siciliano	21	0.881	0.119	18	0.000	1.000

[1]The Italian Large White and Italian Duroc pigs were the same animals as used in the association analyses. All these pigs had genotype CC at the g.1843C>T polymorphic site of the ryanodine receptor 1 (*RYR1*) gene. Allele frequencies of *CTSD* for the Italian Landrace breed have been reported in Russo et al. (2008).

INTRODUCTION

An imprinted QTL with paternal expression affecting muscle mass deposition, carcass fatness, and heart size has been identified in the telomeric end of the p arm of SSC2 (Jeon et al., 1999; Nezer et al., 1999). The presence of QTL in this region was subsequently reported in other studies, confirming imprinted effects in some cases and Mendelian inheritance in other cases (de Koning et al., 2000; Rattink et al., 2000; Evans et al., 2003; Lee et al., 2003; Jungerius et al., 2004; Thomsen et al., 2004; Vidal et al., 2005; Sanchez et al., 2006; van Wijk et al., 2006; Liu et al., 2007, 2008; Tribout et al., 2008). The causative mutation of the paternally expressed QTL was identified in a highly conserved regulatory region of intron 3 of the *IGF2* gene (Van Laere et al., 2003). The *IGF2* intron3-g.3072G>A substitution disrupts a repressor nuclear factor binding site, causing a 3-fold overexpression of postnatal skeletal muscle *IGF2* mRNA in pigs inheriting the mutation from their sires, leading to increased muscle mass and, in turn, reduced backfat deposition (Van Laere et al., 2003).

Recently, we assigned, by linkage mapping, the cathepsin D (***CTSD***) gene on SSC2 close to the *IGF2* region and showed that an SNP (g.70G>A, AM933484) in the 3′-untranslated region (**UTR**) was strongly associated with several carcass and production traits in a sib-tested Italian Large White population (Russo et al., 2008). Because the *IGF2* intron3-g.3072G>A substitution was not analyzed in that study, we could not clearly state whether the effects of the *CTSD* marker were due to linkage disequilibrium with the *IGF2* quantitative trait nucleotide.

To clarify this issue, we investigated the association between both *IGF2* intron3-g.3072G>A and *CTSD* g.70G>A polymorphisms and meat, carcass, and production traits in Italian Large White and Italian Duroc pigs.

MATERIALS AND METHODS

All procedures described were in compliance with Italian and European Union regulations for animal care and slaughter.

Animals

Two groups of pigs were included in the association analysis between DNA markers and EBV or random residuals (**RR**) from fixed models for production and carcass traits and phenotypic measures for meat quality traits. The first group was made up of 270 sib-tested Italian Large White pigs (179 females and 91 castrated males from 79 different sires) already described by Russo et al. (2008). From this group that originally included 272 pigs, 2 pigs were excluded, 1 because it was heterozygous at the ryanodine receptor 1 (***RYR1***) g.1843C>T polymorphic site (Fuji et al., 1991) and another because it was not possible to obtain a genotype for the *IGF2* intron3-g.3072G>A mutation. The second group of pigs was composed of 297 sib-tested Italian Duroc pigs (187 females and 110 castrated males from 135 different sires) slaughtered from 1995 to 2003. The Italian herdbook routinely conducts on-station tests on triplets of littermates (2 females and 1 castrated male). Siblings are performance tested and slaughtered for the genetic evaluation of a boar from the same litter. In addition, a panel of unrelated pigs of different breeds (Table 1), for which no EBV or phenotypic measures were available, was used for allele frequency evaluation.

Performance Test, and Carcass and Meat Quality Traits

The 2 groups of tested pigs used for the association study were performance tested at the test station of the National Association of Pig Breeders. The test period begins when piglets are 30 to 45 d old and ends when they reach 155 ± 5 kg of BW. The nutritive level was quasi ad libitum, meaning that about 60% of pigs were able to ingest the entire supplied ration. Feed intake was recorded daily and BW was measured every 2 wk, and then daily BW gain and the feed:gain ratio (**FGR**) were calculated. At the end of test, selected animals from 2 contiguous batches on trial were mixed at loading and transported to a commercial abattoir located at 24.5 km away from the test station. After unloading, pigs were immediately stunned by CO_2 (concentration

87%) using a dip lift system (Butina, Holbaek, Denmark) and killed by exsaguination in a lying position.

Within 3 h postmortem at the abattoir, backfat thickness (**BFT**) at the musculus gluteus medius, weight of lean cuts (**LC**, necks and loins), and ham weights (**HW**, in Italian Large White pigs only) were measured.

Only for Italian Large White pigs, measures of pH at 2 h postmortem; pH at 24 h postmortem; glycolytic potential, including glycogen and lactate content (30 min postmortem); and cathepsin B activity (24 h postmortem) were obtained on musculus semimembranosus, as described previously (Fontanesi et al., 2008; Russo et al., 2008).

Analysis of DNA Markers

The DNA samples were extracted from blood, lyophilized muscle samples, or hair roots by using standard protocols (Sambrook et al., 1989). The PCR primers designed for analysis of the *IGF2* intron3-g.3072G>A substitution were forward, 5′-GACCGAGCCAGGGACGAG-3′; reverse, 5′-CGCGCCCCACGCGCTCCCACGCTG-3′. The underlined base in the *IGF2* reverse primer is a mismatched nucleotide inserted to create an artificial restriction site for *Ade*I ($CACNNN^{GTG}$). The PCR primers designed for the analysis of the *CTSD* g.70G>A polymorphism were forward, 5′-GCTGTGCACCCTAGGAACC-3′; reverse, 5′-TCGTCAGGTCCAGGACAAAC-3′ (Russo et al., 2008). Polymerase chain reaction was carried out using a PT-100 thermal cycler (MJ Research, Watertown, MA) in a final volume of 20 μL that included 10 pmol of each primer, 2.5 m*M* of $MgSO_4$ (*IGF2*) or 2.5 m*M* of $MgCl_2$ (*CTSD*), 2.5 m*M* each deoxynucleotide 5′-triphosphate, 1 U of EuroTaq DNA polymerase (EuroClone Ltd., Paington, Devon, UK), 0.3× PCRx Enhancer Solution (PCRx Enhancer System, Invitrogen, Carlsbad, CA) only for *IGF2* and 1× PCRx AmpBuffer (PCRx Enhancer System, Invitrogen; *IGF2*) or 1× EuroTaq amplification buffer (*CTSD*). The PCR profiles were as follows: an initial step of denaturation for 5 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 62°C (*IGF2*) or 59°C (*CTSD*), and 30 s at 72°C; the final extension step was for 5 min at 72°C. Mutations were analyzed by PCR-RFLP. Digestion of the *IGF2* amplified fragment of 85 bp with *Ade*I (Fermentas, Vilnius, Lithuania; 5 units in a 25-μL reaction volume containing 5 μL of PCR product and 1× enzyme buffer at 37°C overnight) resulted in 2 fragments of 65 and 20 bp when the g.3072A was present. No digestion occurred in the g.3072G allele. Digestion of the *CTSD* amplicon (184 bp) was obtained with *Msc*I (Fermentas) using the same reaction conditions reported above. The *CTSD* g.70G allele was not cut (184 bp), whereas the g.70A allele resulted in 2 fragments of 117 and 67 bp. The PCR-RFLP products were resolved on 10% polyacrylamide:bis-acrylamide 29:1 gels stained with ethidium bromide.

Radiation Hybrid Mapping of CTSD

The INRA-Minnesota 7,000-rad radiation hybrid (**RH**) panel (Yerle et al., 1998), consisting of 118 rodent-porcine hybrid cell lines, was screened by means of PCR using the same *CTSD* primers and PCR conditions reported above. No PCR fragment was obtained from the control rodent genomic DNA. The PCR reactions were visualized on 10% polyacrylamide:bis-acrylamide 29:1 or 2% agarose gels. The results of RH PCR products were analyzed with the INRA-Minnesota 7,000-rad RH panel mapping tool (accessible through http://imprh.toulouse.inra.fr/; Milan et al., 2000).

Expression Analysis of the CTSD g.70G>A Alleles

Samples of LM were collected at the abattoir from 270 sib-tested Italian Large White pigs (the same animals used in the association study), immediately frozen in liquid N, and stored at −80°C. Total RNA was extracted from 19 muscle samples of pigs heterozygous at the *CTSD* polymorphic site (8 with the GG genotype and 11 with the GA genotype at the *IGF2* mutated site) by using the SV Total RNA Isolation System kit (Promega Corporation, Madison, WI) according to the instructions of the manufacturer. The RNA was treated with DNase I using a RNase-Free DNase Set (Qiagen, Hilden, Germany) as recommended by the manufacturer. The DNase-treated RNA was checked for genomic DNA contamination amplifying a fragment of another gene whose primers were designed on intronic regions (data not shown). From each sample, approximately 1 μg of total RNA was reverse transcribed using an ImProm-II Reverse Transcription System kit (Promega Corporation), as reported in the manual supplied by the manufacturer. The PCR products of 320 bp obtained with primers designed in the 3′-UTR of *CTSD* (forward: 5′-CTGTTCTGTTCCGTGGTGTC-3′; reverse: 5′-CCTTCCTCGTCAGGTCCA-3′) with the same PCR conditions reported above were then sequenced using the same PCR primers and the Big Dye v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequencing reactions, after EDTA, 100% ethanol, and 70% ethanol precipitations, were loaded on an ABI3100 Avant sequencer (Applied Biosystems). Sequencing electropherograms were read with Sequencing Analysis software v. 3.7 (Applied Biosystems). In addition, *CTSD* PCR products were obtained from genomic DNA of 6 heterozygous g.70GA pigs and sequenced as indicated above. These sequencing reactions represented the reference samples with a natural 50:50 ratio of the 2 alleles. Evaluation of possible preferential expression of the 2 *CTSD* alleles in adult skeletal muscle was obtained as a normalized ratio between the sequencing peak height of the 2 bases at the polymorphic site compared with the reference samples, as described in Fontanesi et al. (2009).

Statistical Analyses

The properties of EBV in association studies with DNA markers have been evaluated in several reports, mainly dealing with QTL mapping and association studies in dairy cattle, suggesting a smaller, or at best equivalent, power in using EBV as compared with phenotypic measurements (Thomsen et al., 2001; Israel and Weller, 2002; Viitala et al., 2003; Daetwyler et al., 2008). The use of EBV is convenient in commercial pig breeding stocks because they are routinely calculated by the company or the herdbook. However, depending on the structure of the pig population under investigation, the use of EBV could lead to underestimation of QTL effects (Tribout et al., 2008). An alternative to the use of EBV is the use of phenotypic data. However, to remove the effects of "noisy" environmental fixed factors, RR from analyses of phenotypic data by fixed GLM could be used instead. Because fixed models are capable of removing only the effects of known environmental sources of variation, RR will include all genetic factors (without any assumption regarding their mode of inheritance), permanent environmental factors, and unknown environmental factors together with measurement errors.

The EBV for ADG (expressed in grams), LC (expressed in kilograms), BFT (expressed in millimeters), and FGR were calculated for both groups of sib-tested Italian pigs. The EBV for HW (expressed in kilograms) was calculated for Italian Large White pigs only. The EBV 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, the interaction of sex × age at slaughter, slaughter date, and the random effects of litter and animal.

The RR were calculated for all considered traits in the 2 sib-tested pig populations (ADG, LC, BFT, FGR, and HW in Italian Large White; ADG, LC, BFT, and FGR in Italian Duroc). The RR were obtained by using linear fixed models including the same factors used for each trait in the BLUP multiple-trait animal model.

Because DNA of the parent animals was not available, it was not possible to evaluate the origin of the alleles in the pigs of these 2 groups and to design models including imprinting effects. However, other studies that fitted Mendelian models could identify associations between imprinted genes and phenotypes (Nezer et al., 1999; Cheng et al., 2008).

Association analyses were carried out independently for the 2 groups of sib-tested Italian pigs. Associations between DNA markers and EBV or RR were assessed by using the GLM procedure (SAS Inst. Inc. Cary, NC). 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, the MIXED procedure of SAS was applied to a model that included slaughter date (6 different days at the same abattoir), sex, and the *IGF2* intron3-g.3072G>A genotype as fixed effects and the sire as random effect.

When these models were used, different levels of analysis were considered to isolate the effects of the *IGF2* and *CTSD* markers:

1. The *IGF2* intron3-g.3072G>A polymorphism was analyzed in the Italian Large White pigs (n = 270), considering all 3 genotypes (model 1).
2. To eliminate possible confounding caused by the imprinting effect in the animals with a heterozygous genotype at the *IGF2* locus (g.3072GA), the data were also elaborated by excluding this genotypic class and considering only the 2 homozygous genotypes. This was possible only in the Italian Large White group of pigs (n = 162), which presented enough homozygous genotypes (g.3072GG and g.3072AA) for this elaboration (model 2).
3. In the Italian Duroc pigs (n = 297), an association analysis between the *IGF2* genotypes and either EBV or RR was carried out, excluding the 2 homozygous g.3072GG animals or including them in the heterozygous class (g.3072GA).
4. In the Italian Large White pigs (n = 270), an association analysis between the *CTSD* genotypes and either EBV or RR was carried out, excluding the 4 homozygous g.70GG animals or including them in the heterozygous class (g.70GA; results of the association analysis between EBV and the *CTSD* marker in the Italian Large White pigs have been described by Russo et al., 2008).
5. In the Italian Duroc population tested, only 2 animals with the *CTSD* genotype g.70GG were detected. An association analysis between either EBV or RR and the *CTSD* marker genotypes in this breed was carried out using 2 genotypic classes: one composed of animals with the g.70GG + g.70GA (n = 2 + 42 animals) genotypes or only the g.70GA genotype (the 2 g.70GG animals were excluded), and another with the g.70AA genotype. In addition, an association analysis was carried out by including all animals (n = 297) or excluding pigs that were heterozygous at the *IGF2* polymorphic site (n = 11), or by excluding the only animal that was heterozygous at both the *CTSD* and *IGF2* markers.
6. An association analysis with the *CTSD* marker genotypes in the Italian Large White pigs was carried out for the animals having genotype *IGF2* intron3-g.3072GG (n = 59).

In addition, haplotypes between the *IGF2* and *CTSD* markers were inferred by using the PHASE program v. 2.0 (Stephens et al., 2001). Evaluation of the haplotype

substitution effect on EBV and RR was obtained only in the Italian Large White population (n = 270), using the REG procedure of SAS with a model including the number (0, 1, 2) of the 3 haplotypes available.

The additive genetic effect for the *IGF2* genotypes in the Italian Large White population was estimated as one-half the difference between the EBV or RR of the 2 homozygous groups. The dominance effect at the *IGF2* locus was estimated as the difference between the EBV or RR of the heterozygous group and the average of the EBV or RR of the 2 homozygous groups. Differences from zero of the estimates of additive and dominance effects were tested by *t*-test (Russo et al., 2008).

RESULTS AND DISCUSSION

Allele Frequencies

Allele frequencies of the *IGF2* and *CTSD* markers for the 2 sib-tested pig populations (Italian Large White and Italian Duroc breeds) are shown in Table 1. At the *IGF2* locus, the Italian Large White pigs showed balanced allele frequencies (*IGF2* intron3-g.3072G = 0.419; *IGF2* intron3-g.3072A = 0.581), whereas in the Italian Duroc population, the wild-type g.3072G allele was quite rare (0.025), confirming data already reported for other Duroc populations (Vykoukalová et al., 2006; Yang et al., 2006; Ojeda et al., 2008). Eleven Italian Duroc pigs showed the *IGF2* intron3-g.3072GA genotype and 2 had the intron3-g.3072GG genotype. In the Italian Landrace, Cinta Senese, Casertana, and Nero Siciliano breeds, the *IGF2* intron3-g.3072G allele was fixed or almost fixed, partially confirming for the local breeds the results reported by Ojeda et al. (2008). A wide range of *IGF2* allele frequencies was observed when comparing other Landrace populations (Ojeda et al., 2008). This might reflect different selection strategies and the use of paternal and maternal Landrace lines in crossbreeding schemes. In fact, the *IGF2* intron3-g.3072A allele seems to have negative effects on reproductive performance of the sows caused by excessive leanness (Buys et al., 2006). In both the Italian Large White and Italian Duroc populations, allele g.70G of the *CTSD* gene was less frequent. In the other analyzed breeds, this allele was identified in only 1 Cinta Senese pig and in few Italian Landrace animals.

RH Mapping of CTSD

Linkage mapping of the porcine *CTSD* gene positioned this gene in the telomeric end of the p arm of SSC2 (Russo et al., 2008). However, we wanted to confirm and refine its position to better evaluate its closeness with *IGF2*. The RH mapping indicated that the closest marker obtained by 2-point analysis was *Swc9* (distance = 24 centirads; logarithm of odds = 13.71; retention fraction = 27%), which is a microsatellite in the 3′-UTR of the porcine *IGF2* gene (Nezer et al., 1999). The closeness between *IGF2* and *CTSD* might pose some difficulties in dissecting the effects of both genes when using experimental populations in which artificial linkage disequilibrium is created when crossing divergent lines or breeds. This might be the origin of speculative conclusions in several studies that reported QTL in the SSC2p end and that were not conclusive in distinguishing the presence of different QTL in this region (de Koning et al., 2000; Rattink et al., 2000; Lee et al., 2003; Jungerius et al., 2004; Thomsen et al., 2004; Estellé et al., 2005; Vidal et al., 2005; Sanchez et al., 2006; Liu et al., 2007, 2008; Tribout et al., 2008). Commercial populations, in which it could be possible to identify recombinants between these 2 markers, might represent a suitable material to distinguish their effects.

Assessment of Allele Specific Expression of the CTSD Gene in Skeletal Muscle

Because the *CTSD* gene is located close to the imprinted *IGF2* gene in the SSC2p region, where several reports have identified imprinted and nonimprinted QTL (Jeon et al., 1999; Nezer et al., 1999; de Koning et al., 2000; Rattink et al., 2000; Evans et al., 2003; Lee et al., 2003; Jungerius et al., 2004; Thomsen et al., 2004; Vidal et al., 2005; Sanchez et al., 2006; van Wijk et al., 2006; Liu et al., 2007, 2008; Tribout et al., 2008), we questioned whether allele-specific expression would have affected the *CTSD* gene. In theory, an imprinted gene would exhibit monoallelic expression (hemizygosity) in a parent-of-origin-specific manner, whereas a biallelically expressed gene (not imprinted) would exhibit heterozygosity at a polymorphic site. A significant allelic imbalance from the 50:50 ratio could be the cause of preferential expression (imprinted genes in which both alleles are expressed but where one is expressed more strongly than the other in a parent-of-origin-specific way) or differential allelic expression (allelic expression in which alleles of nonimprinted genes are not expressed equally at the mRNA level; Khatib, 2007). To analyze the expression status of the *CTSD* gene in adult skeletal muscle, we used 19 heterozygous g.70GA Italian Large White pigs and compared the sequenced reverse transcription-PCR products with the sequenced genomic DNA PCR products. In all analyzed samples, both alleles were detected (data not shown), indicating that *CTSD* is not an imprinted gene, at least in adult skeletal muscle. Moreover, we did not note any significant deviation of the 2 alleles from the 50:50 ratio (the estimated transcript ratio was on average 47:53 ± 5 for the G and A alleles, respectively). Therefore, no preferential expression or differential allelic expression affected the *CTSD* gene in the analyzed tissue. Skeletal muscle showed imprinting of the *IGF2* gene both in fetuses and in adult pigs (Nezer et al., 1999; Van Laere et al., 2003; Wrzeska et al., 2006; Li et al., 2008). Both *IGF2* and the close *H19* gene showed imprinting

in several other porcine tissues and also at the embryonic level (Han et al., 2008; Li et al., 2008; Park et al., 2009), similar to other species (Morison et al., 2005). However, biallelic expression of the porcine *CTSD* gene excluded imprinting mechanisms in the skeletal muscle. Even if no report in other species suggests *CTSD* to be an imprinted gene, because the porcine *CTSD* gene seems ubiquitously expressed in pigs (Mei et al., 2008), additional studies should be carried out to confirm its biallelic expression in other tissues.

Association Analyses

We evaluated the effects of the *IGF2* intron3-g.3072G>A SNP in the Italian Large White population, for which no previous data have been reported. Results of the association analyses are shown in Table 2. Significant differences between the 3 genotypes were evident for all EBV and RR considered, with the most significant result being for LC EBV ($P = 2.2 \times 10^{-18}$, model 1). The same significant result ($P < 1.0 \times 10^{-20}$) was obtained after excluding heterozygous animals because we did not know the origin (paternal or maternal) of the g.3072A allele. Differences in genotype effects were more significant with EBV than with RR. This seems to have been due to a reduction in error in the EBV models. In general, we obtained far more significant results than other studies concerning the effects of the *IGF2* alleles on production traits in commercial pig populations (i.e., Estellé et al., 2005; Vykoukalová et al., 2006; Oczkowicz et al., 2009). This could have been due to the use of EBV or RR instead of raw phenotypic measures in the association analysis and possibly to the high quality of performance data. The direction of the effects was consistent with known genetic correlations among traits, with the *IGF2* intron3-g.3072AA genotype showing the greatest LC, ADG, and HW, the least BFT, and the most favorable FGR. The *IGF2* intron3-g.3072GG genotype showed opposite effects. The *IGF2* intron3-g.3072GA genotype was intermediate between the 2 homozygous genotypes. As a consequence, additive effects for all EBV (Table 3) and RR (data not shown) were highly significant. Considering that only the paternally inherited g.3072A allele should be expressed before birth to increase muscle mass (Nezer et al., 1999; Van Laere et al., 2003), least squares means of the heterozygous animals might suggest that one-half of these pigs could have received the g.3072A allele from their father and one-half from their mother, obtaining the averaged observed intermediate values compared with the 2 homozygous genotypes. However, SE (Table 2) and SD (data not shown) were less than expected in the heterozygous group of animals: should a heterozygote g.3072GA pig receive the A allele from its mother, its effect on the response trait would be similar to that of the g.3072GG animals, whereas if the A allele were received from its father, its effect would be similar to that of the g.3072AA animals. Therefore, heterozygotes are expected to show larger variability than each of the 2 homozygous groups. There are 3 possible explanations. The first is that the calculation of both EBV and RR could reduce variability, but this hypothesis is not plausible because different factors and assumptions underlie the calculation of EBV and RR, whereas the 2 analyses showed almost overlapping results. A second hypothesis is that partial postnatal depression of the maternal allele in skeletal muscle (Van Laere et al., 2003) might occur, which has also been observed in other tissues (Wrzeska et al., 2006). In this way, it could explain the superiority of heterozygous animals inheriting the g.3072A from their mothers over the homozygous g.3072GG pigs, reducing variability in the heterozygous class (Van Laere et al., 2003). On the other hand, the supposed partial activity of the maternally inherited g.3072A allele would be compatible with the inferiority of the g.3072GA pigs to the g.3072AA pigs. Gene expression analyses in different tissues and stages would be needed to clarify the role of the maternally inherited g.3072A allele in affecting performance and production traits. A third hypothesis would consider the presence of other polymorphism(s) affecting the analyzed traits in complete or partial linkage disequilibrium with the *IGF2* intron3-g.3072G>A, making the effects of the 2 alleles perfectly additive.

Despite the strong association of the *IGF2* marker with meat, carcass, and production traits, no significant result was observed for the meat quality traits included in this study (Table 2), confirming, to some extent, the results obtained by others (Estellé et al., 2005; Van den Maagdenberg et al., 2007; Oczkowicz et al., 2009).

Association results between the *IGF2* polymorphism and the EBV or RR in the Italian Duroc pigs were not significant (data not shown), probably because of the small number of heterozygous (n = 11) and g.3072GG (n = 2) animals. However, the direction of the effects was the same as observed in the Italian Large White pigs.

To evaluate the effects of the *CTSD* SNP, we first considered the Italian Duroc pigs, excluding an animal that was heterozygous at both the *CTSD* g.70G>A and the *IGF2* intron3-g.3072G>A mutations. (All other Italian Duroc pigs that were g.70AG or g.70GG for the *CTSD* gene carried the *IGF2* intron3-g.3072AA genotype.) Results of the association study in this group, carried out by excluding the 2 *CTSD* g.70GG animals and considering only the *CTSD* g.70AG and g.70AA genotypic classes, are shown in Table 4. Exclusion of all *IGF2* intron3-g.3072GA and GG animals showed almost the same results, as well as separate inclusion of the 2 *CTSD* g.70GG animals or inclusion of them in the *CTSD* g.70AG genotypic class (data not shown). The less frequent genotypic class (*CTSD* g.70AG) showed less ADG, less LC, greater BFT, and less favorable FGR compared with the *CTSD* g.70AA genotype ($P < 0.001$ for all EBV and BFT RR; $P < 0.10$ for all other RR). These results were almost overlapping with the results we obtained in the Italian Large White pigs

Table 2. Association analysis between the *IGF2* intron3-g.3072G>A genotypes and EBV and random residuals (RR) for meat production and carcass traits and phenotypic measures for meat quality variables in Italian Large White pigs (least squares means ± SE)

	Genotype[2]			*P*-value[3]	
Trait[1]	g.3072GG (n = 59)	g.3072GA (n = 108)	g.3072AA (n = 103)	Model 1	Model 2
EBV ADG, g	+13.932 ± 3.035	+30.398 ± 2.243	+47.806 ± 2.298	2.6×10^{-16}	2.7×10^{-14}
EBV LC, kg	+0.584 ± 0.207	+1.758 ± 0.153	+3.063 ± 0.157	2.2×10^{-18}	$<1.0 \times 10^{-20}$
EBV BFT, mm	0.146 ± 0.461	−1.830 ± 0.340	−3.605 ± 0.349	2.2×10^{-9}	1.1×10^{-10}
EBV HW, kg	+0.268 ± 0.075	+0.583 ± 0.056	+0.771 ± 0.057	1.5×10^{-6}	1.6×10^{-7}
EBV FGR	−0.057 ± 0.019	−0.144 ± 0.014	−0.209 ± 0.014	2.3×10^{-9}	2.2×10^{-9}
RR ADG, g	−19.777 ± 10.148	−12.374 ± 7.391	18.094 ± 7.652	0.0028	0.0033
RR LC, kg	−1.547 ± 0.287	−0.458 ± 0.236	1.300 ± 0.216	2.9×10^{-7}	5.9×10^{-14}
RR BFT, mm	2.764 ± 0.619	−0.215 ± 0.462	−1.481 ± 0.467	0.0002	9.8×10^{-8}
RR HW, kg	−0.326 ± 0.156	−0.110 ± 0.122	0.299 ± 0.118	0.0063	0.0017
RR FGR	0.120 ± 0.040	0.038 ± 0.030	−0.055 ± 0.030	0.0028	0.0006
pH_1	5.940 ± 0.033	5.920 ± 0.025	5.930 ± 0.025	0.876	0.907
pH_u	5.673 ± 0.028	5.642 ± 0.022	5.668 ± 0.022	0.534	0.940
Glycogen, μmol	46.186 ± 3.124	48.855 ± 2.408	49.551 ± 2.460	0.650	0.300
Lactate, μmol	58.080 ± 2.045	57.776 ± 1.534	54.870 ± 1.575	0.301	0.163
GP, μmol	104.470 ± 3.173	106.840 ± 2.440	104.230 ± 2.492	0.665	0.829
Catb, nmol	1.176 ± 0.032	1.156 ± 0.024	1.151 ± 0.025	0.803	0.734

[1]LC = lean cuts (kg); BFT = backfat thickness (mm); HW = ham weight (kg); FGR = feed:gain ratio; pH_1 = pH at 2 h postmortem; pH_u = pH at 24 h postmortem; glycogen and lactate (micromoles of lactic acid equivalent per gram of fresh muscle); GP = glycolytic potential (micromoles of lactic acid equivalent per gram of fresh muscle); Catb = cathepsin B activity (nanomoles of 7-amino-4-methylcoumarin released per minute per gram of muscle).

[2]The number of animals for each genotype class was as follows: g.3072GG, n = 59; g.3072GA, n = 108; g.3072AA, n = 103.

[3]Model 1 included all 3 genotypes. Model 2 included only the 2 homozygous genotypes (g.3072GG and g.3072AA). LSM for the 2 genotypes were the same as for model 1.

(Table 4; Russo et al., 2008). However, in the Italian Duroc pigs, no confounding effects could be attributed to the *IGF2* intron3-g.3072G>A mutation.

A second evaluation of the effects of the *CTSD* g.70G>A polymorphism was obtained by considering only Italian Large White pigs homozygous for the *IGF2* intron3-g.3072G allele (n = 59 animals). Animals with this genotype were the only pigs carrying the *CTSD* g.70G allele. Differences between the *CTSD* genotype least squares means of the HW EBV, ADG RR, BFT RR, and LC RR showed $P < 0.05$, and for LC EBV and HW RR showed $P < 0.10$, even in this small group of pigs. The other comparisons were not significant, probably because of the small sample size (Table 5). However, the effect on HW and LC, as well as the differences between least squares means of the other traits, were in the expected direction, with animals carrying the *CTSD* g.70G allele showing less HW, less LC, less ADG, greater BFT, and less favorable FGR as compared with the *CTSD* g.70AA animals. These results supported an effect of the *CTSD* g.70G>A polymorphism independent from the genotype at the *IGF2* intron3-g.3072G>A SNP (Table 4; Russo et al., 2008).

A third evaluation was obtained using the *IGF2* and *CTSD* haplotypes in the Italian Large White population. Three haplotypes were inferred: haplotype [G:G] = *IGF2* intron3-g.3072G and *CTSD* g.70G; haplotype [G:A] = *IGF2* intron3-g.3072G and *CTSD* g.70A; and haplotype [A:A] = *IGF2* intron3-g.3072A and *CTSD* g.70A. In the Italian Duroc group of pigs, only a few haplotypes were represented (data not shown); therefore, this population was not fully informative for an association analysis including haplotypes of these 2 markers. Table 6 shows the estimated regression coefficient of the haplotype substitution effect for the considered EBV and RR. No significant results were obtained for meat quality traits (data not shown), but highly significant results were generally obtained for all EBV and RR. Haplotype [G:G] was associated with reduced ADG, LC, and HW and greater BFT and FGR ($P <$

Table 3. Additive and dominance effects (±SE) obtained for the *IGF2* intron3-g.3072G>A marker in Italian Large White pigs

Trait[1]	Additive effect	*P*-value	Dominance effect	*P*-value
EBV ADG, g	16.937 ± 1.903	<0.0001	−0.471 ± 2.942	0.873
EBV LC, kg	1.240 ± 0.130	<0.0001	−0.066 ± 0.201	0.743
EBV BFT, mm	−1.876 ± 0.288	<0.0001	−0.100 ± 0.446	0.823
EBV HW, kg	0.251 ± 0.047	<0.0001	0.063 ± 0.073	0.387
EBV FGR	−0.076 ± 0.012	<0.0001	−0.011 ± 0.018	0.540

[1]LC = lean cuts (kg); BFT = backfat thickness (mm); HW = ham weight (kg); FGR = feed:gain ratio.

Table 4. Association analysis between the cathepsin D (*CTSD*) g.70G>A genotypes and EBV and random residuals (RR) in Italian Duroc pigs and RR in Italian Large White pigs (least squares means ± SE)

		Genotype[2]		
Breed	Trait[1]	g.70GA (n = 42)	g.70AA (n = 253)	*P*-value
Italian Duroc	EBV ADG, g	+15.419 ± 4.486	+32.996 ± 1.821	0.0008
	EBV LC, kg	+0.867 ± 0.308	+2.158 ± 0.127	0.0001
	EBV BFT, mm	0.286 ± 0.601	−2.196 ± 0.248	0.0002
	EBV FGR	−0.082 ± 0.029	−0.176 ± 0.010	0.0008
	RR ADG, g	−13.045 ± 10.634	6.641 ± 4.384	0.0880
	RR LC, kg	−0.602 ± 0.373	0.153 ± 0.154	0.0625
	RR BFT, mm	2.176 ± 0.769	−0.273 ± 0.317	0.0035
	RR FGR	0.0617 ± 0.043	−0.023 ± 0.018	0.0700
		Genotype[3]		
		g.70GA (n = 36)	g.70AA (n = 230)	
Italian Large White	RR ADG, g	−29.315 ± 12.957	3.044 ± 5.126	0.0210
	RR LC, kg	−1.242 ± 0.440	0.210 ± 0.174	0.0024
	RR BFT, mm	1.729 ± 0.835	−0.424 ± 0.330	0.0172
	RR HW, kg	−0.416 ± 0.215	0.073 ± 0.085	0.0354
	RR FGR	0.148 ± 0.053	−0.005 ± 0.021	0.0079

[1]LC = lean cuts (kg); BFT = backfat thickness (mm); FGR = feed:gain ratio.
[2]The number of animals for each genotype class in the Italian Duroc population was as follows: g.70GA, n = 42; g.70AA, n = 253. Only 2 animals had genotype g.70GG. These pigs were excluded from this analysis.
[3]The number of animals for each genotype class in the Italian Large White population was as follows: g.70GA, n = 36; g.70AA, n = 230. Only 4 animals had genotype g.70GG. These pigs were excluded from this analysis.

0.001 for EBV; $P < 0.01$ for most RR). On the contrary, haplotype [A:A] was associated with greater ADG, LC, and HW and less BFT and FGR ($P < 0.0001$), whereas haplotype [G:A] was intermediate ($P < 0.05$, considering all measures, but ranging from $P < 0.0001$ to $P = 0.0436$).

Overall, these results indicated a separate effect, consistent across Italian Large White and Italian Duroc, of the *CTSD* g.70G>A marker. This confirms the hypothesis that an additional QTL exists in the *IGF2* region of SSC2 (de Koning et al., 2000; Rattink et al., 2000; Lee et al., 2003; Jungerius et al., 2004; Estellé et al., 2005; Sanchez et al., 2006; van Wijk et al., 2006; Liu et al., 2007, 2008; Tribout et al., 2008).

Cathepsin D is an aspartic lysosomal proteinase involved in a broad spectrum of functions, such as protein degradation, apoptosis, and autophagy, and it has been associated with certain pathological conditions such as cancer, Alzheimer's disease, and neuronal ceroid lipofuscinosis (Zaidi et al., 2008). However, it remains to be

Table 5. Association analysis between cathepsin D (*CTSD*) g.70G>A genotypes and EBV and random residuals (RR) in Italian Large White animals with the *IGF2* intron3-g.3072GG genotype (n = 59 pigs; least squares means ± SE)

	Genotype[2]		
Trait[1]	g.70GG + g.70GA (n = 4 + 17)	g.70AA (n = 38)	*P*-value
EBV ADG, g	+9.619 ± 5.141	+16.316 ± 3.822	0.300
EBV LC, kg	+0.182 ± 0.289	+0.806 ± 0.215	0.088
EBV BFT, mm	+0.771 ± 0.668	−0.200 ± 0.497	0.248
EBV HW, kg	+0.039 ± 0.107	+0.394 ± 0.080	0.010
EBV FGR	−0.034 ± 0.029	−0.069 ± 0.022	0.349
RR ADG, g	−41.139 ± 13.097	−7.449 ± 8.915	0.038
RR LC, kg	−2.320 ± 0.464	−1.098 ± 0.316	0.034
RR BFT, mm	4.364 ± 0.998	1.639 ± 0.679	0.028
RR HW, kg	−0.711 ± 0.262	−0.157 ± 0.178	0.086
RR FGR	0.200 ± 0.065	0.071 ± 0.044	0.105

[1]LC = lean cuts (kg); BFT = backfat thickness (mm); HW = ham weight (kg); FGR = feed:gain ratio.
[2]The number of animals for each genotype class was as follows: g.70GG, n = 4; g.70GA, n = 17; g.70AA, n = 38. The 4 animals with genotype g.70GG were included in the genotypic class with the pigs having the g.70GA genotype.

Table 6. Estimated regression coefficients (±SE) of the haplotype substitution effect and the haplotype effects as a proportion of the SD for EBV and random residuals (RR) of 5 production traits in the Italian Large White population

	Haplotype [G:G][2] (n = 48)			Haplotype [G:A][2] (n = 178)			Haplotype [A:A][2] (n = 314)		
Trait[1]	Regression coefficient	*P*-value	Proportion	Regression coefficient	*P*-value	Proportion	Regression coefficient	*P*-value	Proportion
EBV ADG, g	−17.430 ± 3.829	<0.0001	−0.268	−13.666 ± 2.130	<0.0001	−0.365	16.990 ± 1.870	<0.0001	0.485
EBV LC, kg	−1.214 ± 0.266	<0.0001	−0.268	−1.025 ± 0.146	<0.0001	−0.393	1.247 ± 0.128	<0.0001	0.512
EBV BFT, mm	2.037 ± 0.555	0.0003	0.219	1.458 ± 0.315	<0.0001	0.272	−1.864 ± 0.284	<0.0001	−0.372
EBV HW, kg	−0.348 ± 0.088	0.0001	−0.234	−0.164 ± 0.051	0.0015	−0.192	0.244 ± 0.046	<0.0001	0.305
EBV FGR	0.093 ± 0.022	<0.0001	0.247	0.055 ± 0.013	<0.0001	0.254	−0.075 ± 0.011	<0.0001	−0.370
RR ADG, g	−31.639 ± 11.544	0.0065	−0.165	−14.686 ± 6.720	0.0297	−0.133	22.016 ± 6.179	0.0004	0.213
RR LC, kg	−1.412 ± 0.393	0.0004	−0.215	−1.304 ± 0.219	<0.0001	−0.342	1.547 ± 0.196	<0.0001	0.435
RR BFT, mm	1.970 ± 0.746	0.0088	0.159	1.519 ± 0.428	0.0005	0.212	−1.897 ± 0.392	<0.0001	−0.284
RR HW, kg	−0.479 ± 0.191	0.0129	−0.152	−0.255 ± 0.111	0.0224	−0.139	0.361 ± 0.102	0.0005	0.212
RR FGR	0.148 ± 0.047	0.0019	0.188	0.056 ± 0.028	0.0436	0.123	−0.092 ± 0.025	0.0003	−0.217

[1]LC = lean cuts (kg); BFT = backfat thickness (mm); HW = ham weight (kg); FGR = feed:gain ratio.
[2]Haplotypes are indicated as follows: [G:G] = *IGF2* intron3-g.3072G and *CTSD* g.70G; [G:A] = *IGF2* intron3-g.3072G and *CTSD* g.70A; [A:A] = *IGF2* intron3-g.3072A and *CTSD* g.70A.

clarified whether the *CTSD* gene is directly involved in affecting the traits examined in this study or whether mutation(s) in (an)other close gene(s) in this region could possibly be the causative factor(s) determining greater ADG, greater muscle mass, and less backfat deposition. Bioinformatic predictions did not strongly support a putative function of the 3′-UTR *CTSD* nucleotide transition or by the surrounding nucleotides (data not shown). Functional studies should be carried out to verify this issue. In this study, the porcine *CTSD* gene did not appear to be imprinted in adult skeletal muscle. Therefore, this gene should follow a Mendelian inheritance model, with putative additive effects (data not shown). This could be one of the reasons why the positive *CTSD* allele is the most frequent both in Italian Duroc and in Italian Large White breeds. Even if the selection goals for these 2 breeds take into account several meat quality traits, these populations have still undergone intense family selection for almost 20 yr, based on BLUP EBV that were probably very efficient in selecting the g.70A allele.

In conclusion, our results indicate that the *IGF2* intron3-g.3072G>A mutation is not the only quantitative trait nucleotide affecting production traits in the telomeric end of the p arm of SSC2, as demonstrated by the analysis of the *CTSD* g.70G>A polymorphism. Marker-assisted selection already applied using the *IGF2* mutation (Van Laere et al., 2003; Buys et al., 2006) could increase in efficiency by adding information from the *CTSD* genotype. To this aim, it would be interesting to analyze the combined effects of these 2 polymorphic sites in other breeds and lines. For all practical applications, however, the different modes of inheritance of the 2 genes have to be taken into account.

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