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Single nucleotide polymorphisms in several porcine cathepsin genes are associated with growth, carcass, and production traits in Italian Large White pigs[1]

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ABSTRACT: To identify DNA markers associated with performance, carcass, and meat production traits including muscle postmortem cathepsin activity, several porcine genes encoding for lysosomal proteinases (cathepsin B, *CTSB*; cathepsin D, *CTSD*; cathepsin F, *CTSF*; cathepsin H, *CTSH*; cathepsin L, *CTSL*; and cathepsin Z, *CTSZ*) and for a cathepsin inhibitor (cystatin B) were investigated. Single nucleotide polymorphisms were identified in *CTSD*, *CTSH*, *CTSL*, and *CTSZ* genes with a combination of in silico expressed sequence tag database mining and single-strand conformation polymorphism analysis. Sequencing and PCR-RFLP protocols were used to validate the identified polymorphisms. Allele frequencies at these loci were investigated in Italian Large White, Landrace, Duroc, Piétrain, Belgian Landrace, Hampshire, and Meishan breeds. Genotyping *CTSD* and *CTSH* markers made it possible to genetically map these genes to SSC 2 and 7, respectively. Markers in *CTSD*, *CTSH*, *CTSL*, and *CTSZ* genes, together with mutations we previously reported in cystatin B, *CTSB*, and *CTSF* genes, were genotyped in an Italian Large White sib-tested population (272 or 482 animals). For these animals, meat quality traits (cathepsin B activity, pH measured at 2 h postmortem, pH measured at 24 h postmortem, glycogen, lactate, and glycolytic potential of semimembranosus muscle) and EBV for ADG, lean cuts (LC), backfat thickness (BFT), ham weight (HW), and feed:gain ratio (FGR) were determined. Analyzed markers did not show any association with muscle cathepsin B activity. Thus, it could be possible that different genes, other than these investigated candidates, affect this trait, which is correlated with the excessive softness defect of dry-cured hams. The results of association analysis confirmed the effects we already reported in another study for *CTSF* on ADG ($P = 0.008$), LC ($P = 0.001$), and BFT ($P = 0.02$). Moreover, *CTSD* was associated with ADG, LC ($P < 0.0001$), BFT, HW, and FGR ($P < 0.001$); *CTSH* was associated with FGR ($P = 0.026$); and *CTSZ* was associated with ADG ($P = 0.006$), LC ($P = 0.01$), HW ($P = 0.024$), and FGR ($P = 0.029$). The biochemical and physiological functions of the lysosomal proteinases, together with the results obtained in our investigation, suggest that the cathepsin gene family might play important roles affecting economic traits in pigs.

Key words: candidate gene, cathepsin, cystatin, meat production, pig, single nucleotide polymorphism

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INTRODUCTION

Porcine skeletal muscle cathepsins and their inhibitors (cystatins) have been mainly investigated in relation to muscle transformation during dry-cured ham processing in which residual cathepsin activity is maintained up to 12 to 18 mo postmortem (Toldrá and Etherington, 1988; Parolari et al., 1994; Parreño et al., 1994). Dry-cured ham defects occur when high lysosomal cathepsin activity throughout the processing period causes excessive softness of the final products (Virgili et al., 1995a,b, 1998). Russo et al. (2000) estimated the heritability of muscular cathepsin B activity in Italian Large White pigs, indicating that genetic aspects contribute to determine proteinase-related meat quality variables important for dry-cured ham production. Thus, starting from a candidate gene approach, we investigated

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porcine cathepsin B (***CTSB***) and cystatin B (***CSTB***) genes to identify DNA markers associated with muscle cathepsin activity and other production traits (Russo et al., 2002). The identified mutations did not affect lysosomal proteinase activities. However, *CSTB* was significantly associated with ADG, and *CTSB* was significantly associated with backfat thickness (**BFT**) in Italian heavy pigs of Large White, Duroc, and Landrace breeds. In a following study carried out to verify these indications, the results for *CSTB* were confirmed in an Italian Large White population (Russo et al., 2003). In the same study, a missense mutation in the cathepsin F (***CTSF***) gene (Russo et al., 2004) was significantly associated with ADG, lean meat content, BFT, and feed conversion rate.

Here, we identified mutations in other porcine cathepsin genes (cathepsin D, ***CTSD***; cathepsin H, ***CTSH***; cathepsin L, ***CTSL***; and cathepsin Z, ***CTSZ***) and analyzed these DNA markers, as well as the markers already reported for *CSTB*, *CTSB*, and *CTSF* in a different Italian Large White sib-tested population [combining for the 3 latter genes the data previously obtained by Russo et al. (2003)] for which meat quality traits and several EBV were determined.

MATERIALS AND METHODS

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

Animals

Sib-tested Italian Large White pigs were used for association analysis with DNA markers as described below. These pigs were from 2 groups of animals slaughtered in 1999 to 2000 and 2002, respectively. The first group was constituted by 210 pigs (108 females and 102 castrated males) slaughtered on 18 different days in the same abattoir. The second group was constituted by 180 females and 92 castrated males slaughtered on 6 different days in the same abattoir as the first group. These 2 groups of pigs were different from the pigs analyzed by Russo et al. (2002). The first group of animals was the same as reported by Russo et al. (2003). The structure of the sib-tested Italian Large White population is based on triplets of pigs of the same litter (2 females and 1 castrated male) that are performance-tested and slaughtered for the genetic evaluation of a boar of the same litter. The same structure was in general present in the analyzed pigs of the second group of Italian Large White animals (originated from 80 different sires), whereas for the first group, in most cases, only 2 pigs of the sib-tested triplet (1 female and 1 castrated male, obtained from 102 different sires) were sampled. These 2 populations were used for EBV calculation of performance and carcass traits and for meat quality measurements (see below). A random sample of 30 animals of the second group of Italian Large White pigs was used to confirm the presence of in silico-identified SNP.

A panel of 40 unrelated pigs of different breeds (10 Large White, 10 Duroc, 5 Landrace, 5 Piétrain, 5 Belgian Landrace, 3 Hampshire, and 2 Meishan), sampled in different periods, was used for PCR-single strand conformation polymorphism (**PCR-SSCP**) analysis for the *CTSL* gene. Another panel of unrelated pigs of Landrace, Duroc, Piétrain, Belgian Landrace, Hampshire, and Meishan breeds (Table 1), sampled as described for the 40 animals used for PCR-SSCP analysis, was used for allele frequency evaluation. For these animals and for the 40 pigs reported above, performance, carcass, and meat quality traits were not available. The reference populations of the PiGMaP project (Archibald et al., 1995) were used for linkage mapping of *CTSD* and *CTSH* markers.

Performance Test, Carcass and Meat Quality Traits

The 2 groups of Italian Large White pigs used for the association study were performance-tested at the test station of the National Association of Pig Breeders. The test period of the animals began at approximately 30 kg of BW and ended at 155 ± 5 kg of BW. The nutritive level was quasi ad libitum. Feed intake was recorded daily, BW was measured every 2 mo, and then daily gain and feed:gain ratio (**FGR**) were calculated. At the end of the test, the animals of the same testing period were mixed at loading and transported to a commercial slaughterhouse located 24.5 km from the test station. After unloading, the pigs were immediately stunned by CO_2 (concentration 87%) using a dip lift system (Butina, Holbæk, Denmark) and bled in a lying position.

At the slaughterhouse, within 3 h postmortem, BFT at the level of musculus gluteus medius, weight of lean cuts (**LC**, necks and loins), and weight of hams were measured. Measures of pH at 2 h postmortem ($\mathbf{pH_1}$) and at 24 h postmortem ($\mathbf{pH_u}$) were determined on musculus semimembranosus using a Crison pH meter equipped with an Ingold Xerolite electrode (Mettler Toledo, Udorf, Switzerland). For glycolytic potential (**GP**) determination, samples of the same muscle were collected at 30 min postmortem and immediately frozen in liquid nitrogen and later freeze-dried. This variable was measured according to Monin et al. (1987) and Nanni Costa et al. (2008) separately determining lactate content and the sum of glycogen, glucose, and glucose-6-phosphate. Briefly, lactate content was measured using the L-Lactic acid BioAnalysis kit (Boehringer Mannheim/R-Biopharma, R-Biopharma GmbH, Darmstadt, Germany). Glycogen was first degraded to glucose with amyloglucosidase from *Aspergillus niger*. Then, the degradation product, muscle glucose, and glucose-6-phosphate were determined using the D-Glucose

Table 1. Allele frequencies for the cathepsin B (*CTSB*), cathepsin D (*CTSD*), cathepsin F (*CTSF*), cathepsin H (*CTSH*), cathepsin L (*CTSL*), cathepsin Z (*CTSZ*), and cystatin B (*CSTB*) markers observed in Italian Large White (LW), Landrace (L), Duroc (D), Piétrain (P), Belgian Landrace (BL), Hampshire (H), and Meishan (M) pigs

		Allele frequencies, no. of animals						
Genes	Alleles	LW[4]	L	D	P	BL	H	M
CTSB[1]	g.72A	0.944	0.943	0.877	0.953	0.911	0.909	1.000
	g.72C	0.056	0.057	0.123	0.047	0.089	0.091	0.000
		(454)	(53)	(53)	(32)	(28)	(22)	(14)
CTSD	g.70G	0.148	0.065	0.125	0.048	0.025	0.000	0.786
	g.70A	0.852	0.935	0.875	0.952	0.975	1.000	0.214
		(271)	(23)	(20)	(21)	(20)	(20)	(7)
CTSF[2]	g.22G	0.306	0.554	0.630	0.444	0.684	0.375	0.056
	g.22C	0.694	0.446	0.370	0.556	0.316	0.625	0.944
		(481)	(46)	(50)	(9)	(19)	(12)	(9)
CTSH	g.122A	0.847	0.857	1.000	1.000	1.000	1.000	1.000
	g.122G	0.153	0.143	0.000	0.000	0.000	0.000	0.000
		(271)	(49)	(41)	(21)	(16)	(21)	(5)
CTSL	g.[32C;34T]	0.996	0.955	0.554	1.000	0.763	1.000	1.000
	g.[32C;34C]	0.000	0.000	0.339	0.000	0.237	0.000	0.000
	g.[32T;34T]	0.004	0.045	0.107	0.000	0.000	0.000	0.000
		(271)	(28)	(33)	(25)	(19)	(24)	(11)
CTSZ	g.37A	0.446	0.925	0.083	0.765	0.625	0.450	1.000
	g.37G	0.554	0.075	0.917	0.235	0.375	0.550	0.000
		(271)	(20)	(30)	(17)	(20)	(20)	(12)
CSTB[3]	g.173A	0.026	0.123	0.052	0.135	0.259	0.222	1.000
	g.173G	0.974	0.877	0.948	0.865	0.741	0.778	0.000
		(481)	(57)	(58)	(48)	(29)	(27)	(14)

[1]Already reported in Russo et al. (2002). Frequencies of alleles 1 and 2 of Russo et al. (2002) have been combined in g.72A.
[2]Already reported in Russo et al. (2004).
[3]Already reported in Russo et al. (2002); g.173A: European Molecular Biology Laboratory accession number AJ315562; g.173G: AJ315563.
[4]Allele frequencies in the Italian Large White breed were determined on the analyzed sib-tested pigs reported in Tables 4 and 5.

Enzymatic BioAnalysis kit (Boehringer Mannheim/R-Biopharma). Glycolytic potential was calculated as the sum of: 2[glycogen + glucose + glucose-6-phoshate] + [lactate] according to Monin and Sellier (1985) and expressed as micromoles of lactic acid equivalent per gram of fresh muscle.

Cathepsin B activity (**CATB**) was analyzed using a sample of musculus semimembranosus collected at 24 h postmortem on the left hams of the slaughtered pigs. Cathepsin B activity was assayed by the method described by Parolari et al. (1994) using N-CBZ-L-arginyl-L-arginine as fluorescent substrate. The enzyme activity was expressed in terms of nanomoles of 7-amino-4-methylcoumarin released per minute per gram of muscle. Cathepsin B activity was measured in both sib-tested Italian Large White groups (210 + 272 pigs), whereas pH_1, pH_u, and GP were measured only in the second group (272 animals). Means, SD, and minimum and maximum values for the meat quality traits are reported in Table 2.

Identification and Analysis of DNA Markers

An in silico SNP search was carried out to identify new DNA markers in porcine *CTSD*, *CTSH*, and *CTSZ* genes mining porcine expressed sequence tags (**EST**) with BLASTN and BLASTX algorithms at the National Center for Biotechnology Information GenBank database (http://www.ncbi.nlm.nih.gov) and the corresponding human cDNA sequences. Then, matched porcine EST sequences (>80% identities) were aligned and manually assembled. Putative SNP were declared when at least 3 aligned different EST sequences were identified for each of 2 bases at the same position.

Search of mutations in the 3′-untranslated region (**UTR**) of the porcine *CTSL* gene was performed by PCR-SSCP analysis as described by Fontanesi et al. (2001a) in a panel of 40 pigs of different breeds reported above. Mutations in the *CSTB*, *CTSB*, and *CTSF* genes were previously reported by Russo et al. (2002, 2004). The mutation in the *CSTB* gene is a missense mutation [D63N; g.173G>A, European Molecular Biology Laboratory (**EMBL**) accession number AJ315563 and AJ315562] identified in exon 3. This mutation was analyzed by PCR-RFLP as described previously (Russo et al., 2002; Table 3). The mutation analyzed in intron 6 of the *CTSB* gene, that differentiates allele 3 (g.72C, EMBL accession number AJ315560) from the other detected alleles (g.72A, alleles 1 and 2; EMBL accession numbers AJ315558 and AJ315559), was chosen because in our previous investigation it was associated

Table 2. Means, SD (in parentheses), minimum (MIN), and maximum (MAX) values for meat quality traits and EBV

Traits[1]	Number of animals	Mean (SD)	MIN	MAX
pH_1	272	5.941 (0.240)	5.41	6.80
pH_u	272	5.669 (0.212)	5.30	6.54
Lactate, μmol/g	272	56.374 (15.632)	23.28	116.94
Glycogen, μmol/g	272	47.123 (22.584)	7.33	135.74
GP, μmol/g	272	103.501 (22.981)	49.23	182.73
CATB, nmol/min/g	272	1.156 (0.226)	0.60	2.00
CATB, nmol/min/g	482	1.443 (0.447)	0.60	3.19
ADG EBV, g	272	+33.321 (26.590)	−65.00	+115.00
ADG EBV, g	482	+31.977 (27.900)	−65.00	+119.00
LC EBV, kg	272	+1.981 (1.861)	−2.94	+8.19
LC EBV, kg	482	+1.866 (1.901)	−2.94	+8.19
BFT EBV, mm	272	−2.044 (3.867)	−12.80	+11.30
BFT EBV, mm	482	−2.300 (3.908)	−12.80	+11.30
HW EBV, kg	272	+0.582 (0.612)	−1.80	+2.63
FGR EBV	272	−0.151 (0.152)	−0.64	+0.48

[1]The markers were genotyped for 272 pigs (second group of sib-tested animals) for which all considered traits were available. For ADG, LC, BFT, and CATB, data were available for an additional 210 pigs (first group of sib-tested pigs) that were genotyped for CATB (*CTSB*), cathepsin F (*CTSF*), and cystatin B (*CSTB*) by Russo et al. (2003), for a total of 482 pigs. pH_1 = pH measured at 2 h postmortem on musculus semimembranosus; pH_u = pH measured at 24 h postmortem on the same muscle; GP = glycolytic potential; CATB = cathepsin B activity; LC = lean cuts; BFT = backfat thickness; HW = ham weight; FGR = feed:gain ratio.

with greater BFT (Russo et al., 2002). A new PCR-RFLP assay was designed to genotype this SNP (Table 3). The *CTSF* DNA marker is a missense mutation identified in exon 9 (D355E; g.22C>G, EMBL accession numbers AM933487 and AM933486, respectively) in a highly conserved region of the protein. This mutation was genotyped by PCR-RFLP as already reported (Russo et al., 2004; Table 3).

The ryanodine receptor 1 (***RYR1***) g.1843C>T polymorphic site (Fujii et al., 1991) was analyzed with a DNA test using the PCR-RFLP method described in Russo et al. (1993). This marker was analyzed to eventually exclude from the association analysis pigs carrying the g.1843T allele, which has a large impact on meat quality, including pH_1, and meat deposition traits (de Vries et al., 1998; Sellier, 1998).

Table 3. Polymerase chain reaction primers, PCR conditions, and PCR-RFLP patterns of the different alleles

Genes	Forward (F) and reverse (R) primers (5′-3′)[1]	PCR[2]	Use[3]
Cathepsin B (*CTSB*)	F: GTGGCCGGGTGGGTTTTA R: TCCTCCTGGTGCTGCTAATTCTGAC	139/55/2.0	PCR-RFLP (*Msp*I): allele g.72A, 108 + 31 bp; allele g.72C, 84 + 31 + 24 bp
Cathepsin D (*CTSD*)	F: GCTGTGCACCCTAGGAACC R: TCGTCAGGTCCAGGACAAAC	184/59/2.5	Sequencing; PCR-RFLP (*Msc*I): allele g.70G, 184 bp; allele g.70A, 117 + 67 bp
Cathepsin F (*CTSF*)[4]	F: AGGGAGGGCTGGAGACGGAGTA R: TCATTCTGGCTCAGCTCCAC	118/58/2.5	PCR-RFLP (*Rsa*I): allele g.G, 118 bp; allele g.C, 97 + 21 bp
Cathepsin H (*CTSH*)	F: AATCTTGCCCTGGAGGAAGT R: GGTTAAAAATCACGCCCAAG	177/58/3.0	Sequencing; PCR-RFLP (*Bst*UI): allele g.122A, 177 bp; allele g.122G, 120 + 57 bp
Cathepsin L (*CTSL*)[5]	F: GATGGTGAGAATAGAGGAC R: AGAGTTAAGCAATGAATCTTC	216/55/3.0	PCR-single strand conformation polymorphism; sequencing; PCR-RFLP (*Hpy*188III): allele g.[32C;34C], 216 bp; alleles g.[32C;34T] and g.[32T;34T], 180 + 36 bp
	F: ATGGTGAGAATAGAGGACCTGAGGACAGTA R: AGAGTTAAGCAATGAATCTTC	215/58/2.5	PCR-RFLP (*Rsa*I): allele g.[32T;34T], 215 bp; alleles g.[32C;34C] and g.[32C;34T], 186 + 29 bp
Cathepsin Z (*CTSZ*)	F: GGCCTCATGAGTACCTGTCC R: ATGTGCTGGTTCCTGGTGAC	100/59/2.5	Sequencing; PCR-RFLP (*Scr*FI): allele g.37A, 100 bp; allele g.37G, 65 + 35 bp
Cystatin B (*CSTB*)[6]	F: GTTCCAGGTTCAAGTTGACGATGTC R: GGTCTGGTAGCTGGACAAGG	100/58/2.5	PCR-RFLP (*Taq*I): allele g.173A, 100 bp; allele g.173G, 75 + 25 bp

[1]Underlined bases in the *CTSF*, *CTSL*, and *CSTB* forward primers are mismatched nucleotides inserted to create artificial restriction sites for *Rsa*I, *Rsa*I, and *Taq*I, respectively.

[2]Product length (bp)/annealing temperature (°C)/[$MgCl_2$].

[3]Use of PCR primers. Restriction patterns are reported for the different alleles.

[4]Polymerase chain reaction primers reported in Russo et al. (2004).

[5]Polymerase chain reaction primers that amplify a fragment of 216 bp are from Fontanesi et al. (2001b). A second PCR primer pair was designed with a mismatch in the forward primer to create an artificial restriction site in the amplified product of 215 bp for alleles g.[32C;34C] and g.[32C;34T].

[6]Polymerase chain reaction primers reported in Russo et al. (2002).

Deoxyribonucleic acid was extracted from blood, lyophilized muscle samples, or hair roots using standard protocols. Polymerase chain reaction was carried out using a PT-100 (MJ Research, Watertown, MA) thermal cycler in a final volume of 20 μL that included 10 pmol of each primer, 2.0 m*M* $MgCl_2$, 2.5 m*M* each deoxynucleoside triphosphate, and 1 U of EuroTaq (EuroClone Ltd., Paington, Devon, UK) DNA polymerase. The PCR profile was the following: an initial step of denaturation for 5 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at the specific annealing temperature for each primer pair (Table 3) and 30 s at 72°C; and the final extension step was for 5 min at 72°C. Sequencing was carried out to confirm the in silico-identified mutations (*CTSD*, *CTSH*, and *CTSZ* genes) and to characterize the *CTSL* SSCP alleles. Sequencing reactions were produced for ExoSAP-IT (USB Corporation, Cleveland, OH)-treated PCR products obtained from 2 pigs of each genotype identified by PCR-RFLP or PCR-SSCP using the same PCR primers (Table 3) and the Big Dye v3.1 kit (Applied Biosystems, Foster City, CA). Sequencing reactions, after a purification step with DyeEx 2.0 Spin columns (Qiagen, Hilden, Germany), were loaded on an ABI3100 Avant sequencer (Applied Biosystems). Mutations were analyzed by PCR-RFLP using 5 μL of PCR product digested overnight at 37 or 65°C with 3 units of the restriction enzymes reported in Table 3 in a final volume of 25 μL containing 1× enzyme reaction buffer. The PCR-RFLP products were resolved on 10% polyacrylamide-bis-acrylamide 29:1 gels stained with ethidium bromide.

The DNA samples belonging to pigs of 3-generation families of the PiGMaP Consortium (Archibald et al., 1995) were genotyped for the polymorphisms identified at the *CTSD* and *CTSH* loci. Samples of unrelated Landrace, Duroc, Piétrain, Belgian Landrace, Hampshire, and Meishan pigs (Table 1) were genotyped for *CTSD*, *CTSH*, *CTSL*, and *CTSZ* markers to obtain allele frequency information in different breeds. For *CTSB*, *CTSF*, and *CSTB* markers, allele frequencies in the same breeds were already reported by Russo et al. (2002, 2004). Mutations at the *CSTB*, *CTSB*, and *CTSF* loci were already genotyped in the first group of sib-tested Italian Large White pigs (210 animals; Russo et al., 2003). Mutations at the *CSTB*, *CTSB*, *CTSD*, *CTSF*, *CTSH*, *CTSL*, and *CTSZ* loci were genotyped on the second group of sib-tested Italian Large White pigs (272 animals). All sib-tested Italian Large White animals were also genotyped for the *RYR1* mutation to exclude carriers of the g.1843T allele.

In Silico Analysis of Missense Mutations

In silico functional analysis of missense mutations identified in *CTSF*, *CTSZ*, and *CSTB* genes was obtained using PANTHER (Thomas et al., 2003), whose predictions have been experimentally validated (Brunham et al., 2005). PANTHER estimates the likelihood of a particular nonsynonymous (AA changing) coding SNP to cause a functional effect on the protein. It calculates the substitution position-specific evolutionary conservation (**subPSEC**) score based on an alignment of evolutionarily related proteins (Thomas et al., 2003; Thomas and Kejariwal, 2004). The probability that a given variant will cause a deleterious effect on protein function is estimated by $P_{deleterious}$, such that a subPSEC score of −3 corresponds to a $P_{deleterious}$ of 0.5 (Brunham et al., 2005). The subPSEC score is the negative logarithm of the probability ratio of the wild-type and mutant AA at a particular position. PANTHER subPSEC scores are continuous values from 0 (neutral) to about −10 (most likely to be deleterious).

Statistical Analysis

Linkage analysis of the *CTSD* and *CTSH* markers was obtained merging the genotypes of these genes with those already genotyped in the PiGMaP reference populations (Archibald et al., 1995). Twopoint and multipoint procedures of the CRI-MAP package version 2.4 (Green et al., 1990) were performed. Multipoint sex-averaged maps were constructed using options ALL, BUILD, CHROMPIC, and FLIPS2–6.

Haplotypes between *CTSD* and *CTSF* genes located on the same chromosome were inferred using the PHASE program v. 2.0 (Stephens et al., 2001) in the second group of sib-tested Italian Large White pigs (272 animals). Linkage disequilibrium between the *CTSD* and *CTSF* markers that are located on the p end of SSC 2 was evaluated for the Italian Large White sib-tested pigs using the 2LD software (Zhao, 2004).

Estimated breeding values for ADG (expressed in g), LC (expressed in kg), and BFT (expressed in mm) were calculated for the first group of sib-tested Italian Large White pigs. The same EBV, as well as EBV for ham weight (expressed in kg, **HW**) and FGR, were computed for the second group of sib-tested Italian Large White pigs. Estimated breeding values were calculated using a BLUP-multiple trait animal model. The model included the fixed effects of batch tested and sex and the random effects of litter, individual permanent environment, animal, age at the beginning of test, age of sow, BW at slaughter, age at slaughter, and inbreeding coefficient (Russo et al., 2000). Means and measures of variability of the considered EBV are reported in Table 2.

Associations between the genotypes of candidate genes and meat quality variables (pH_1, pH_u, glycogen and lactate content, GP, and CATB) were assessed using the MIXED procedure (SAS Institute Inc., Cary, NC) with a model that included the sire as a random effect and the fixed effects of day of slaughtering, sex, and the genotype of the analyzed DNA markers (1 marker for each model run). The GLM procedure (SAS Institute Inc.), was used to test associations between the same genotypes of the considered markers and the EBV cal-

culated for ADG, LC, BFT, HW, and FGR. The model included only the fixed effects of the marker genotypes analyzed individually. All other factors contributing to the variability of the investigated traits were already considered in the calculation of their EBV.

Haplotypes inferred between *CTSD* and *CTSF* genes in the second group of sib-tested Italian Large White pigs (272 animals) were analyzed considering animals having 0, 1, or 2 copies of the haplotype in question using the 2 models applied for the single marker association tests. Additive genetic effect for the *CTSF* and *CTSZ* loci was estimated as half of the difference between the 2 homozygous groups: $a = 1/2(\mathrm{BB} - \mathrm{AA})$, where A and B indicate the first and second allele of the analyzed markers, respectively. The dominance effect was estimated as the difference between the heterozygous group and the average of the 2 homozygous groups at each locus: $d = \mathrm{AB} - 1/2(\mathrm{AA} + \mathrm{BB})$. The estimates of effects were tested by *t*-test on significant deviation from zero.

With 6 genes and 11 traits, there were a total of 66 statistical single marker-trait tests. Thus, attention to the effect of multiple tests on nominal comparison-wise error rate *P*-values is required to evaluate statistical significance of nominal *P*-values. Technically, the classical Bonferroni correction is not appropriate in the present case, because some of the traits are not independent. For example, BFT is negatively correlated with ADG and LC; GP is determined by the sum of lactate content and glycogen content, and so on. Of greater importance, as has been discussed extensively (Weller et al., 1998; Lee et al., 2002; Fernando et al., 2004), the Bonferroni attempt to control the likelihood of including even a single false positive among the tests that are declared significant results in exceedingly stringent threshold *P*-values for significance. Consequently, power is greatly decreased and many potentially useful significant effects are lost. For this reason, approaches based on controlling the false discovery rate (Weller et al., 1998) or proportion of false positives (**PFP**; Fernando et al., 2004) are becoming more widely accepted. The false discovery rate approach is appropriate when there is no a priori information indicating the presence of false null hypotheses among the tests. The PFP approach is appropriate when prior information indicates the presence of at least some false null hypotheses among the tests. In the present case, *CTSB*, *CTSF*, and *CSTB* have been already genotyped in different Italian Large White populations showing significant results (Russo et al., 2002, 2003); results for *CTSF* have been also confirmed in another study (Ramos et al., 2008), and *CTSD*, *CTSH*, and *CTSZ* map near known QTL regions for carcass and meat production and quality traits (see Results and Discussion sections). The PFP thresholds were calculated as described in Bagnato et al. (2008); following their example, we too have adopted a PFP threshold of $P = 0.10$ to declare significance for single marker-trait tests.

RESULTS

Identification of Mutations in Candidate Genes and Allele Frequencies

In silico analysis identified 7 putative SNP in the 3′-UTR of the *CTSD* gene (data not shown), 3 of them created-disrupted restriction sites for endonucleases *Dde*I (2 sites) and *Msc*I. Restriction analysis of 30 Italian Large White pigs did not reveal any polymorphism for the 2 *Dde*I restriction sites, whereas the *Msc*I polymorphism was confirmed in the same animals (Figure 1a). Sequencing of the amplified fragment in animals of different PCR-RFLP genotypes further confirmed the in silico results for this polymorphic site (alleles g.70A and g.70G; EMBL accession numbers AM933485 and AM933484, respectively).

A BLASTN analysis identified 5 putative synonymous SNP in the coding sequence (**CDS**) and 4 in the 3′-UTR of the *CTSH* gene (data not shown). Only 1 of the 3′-UTR putative SNP created-disrupted a palindrome for *Bst*UI. Polymerase chain reaction-RFLP analysis confirmed the presence of this polymorphism in the Italian Large White breed (Figure 1b). Sequencing revealed that the analyzed SNP (g.122A>G) was in linkage disequilibrium with another in silico-detected SNP of the 3′-UTR (g.78G>C). Sequences of the 2 alleles have been deposited in the EMBL database with accession numbers AM933488 and AM933489, respectively.

In silico investigation of porcine *CTSZ* EST sequences revealed 8 putative SNP (7 in the CDS and 1 in the 3′-UTR) and 1 indel (3′-UTR; data not shown). Of the putative SNP in the CDS, 3 were missense mutations (L10P, K65R, and N210I). One of them (K65R, g.37A>G) created-disrupted a restriction site for *Scr*FI that was confirmed in Italian Large White pigs by PCR-RFLP (Figure 1c) and sequencing (EMBL accession numbers AM933493 and AM933494, respectively).

Polymerase chain reaction-SSCP analysis of the porcine *CTSL* 3′-UTR showed the presence of 3 different alleles (Figure 1d) caused by 2 SNP (g.32C>T; 34C>T; EMBL accession numbers AM933490, AM933491, and AM933492). Then, 2 PCR-RFLP protocols were designed to analyze the 2 SNP positions (Figures 1e and 1f; Table 3).

Allele frequencies of the identified polymorphisms at the *CTSD*, *CTSH*, *CTSL*, and *CTSZ* loci were analyzed in Landrace, Duroc, Piétrain, Belgian Landrace, Hampshire, and Meishan breeds and are included in Table 1 together with allele frequencies already reported for the *CTSB*, *CTSF*, and *CSTB* loci (Russo et al., 2002, 2004). Allele frequencies in the Italian Large White breed were obtained from the analysis of the sib-tested pigs and are included in Table 1. For the *CTSB* gene, in this study, we analyzed only the SNP positions that differentiate allele 3 (g.72C, EMBL accession number AJ315560) from the other alleles detected at this lo-

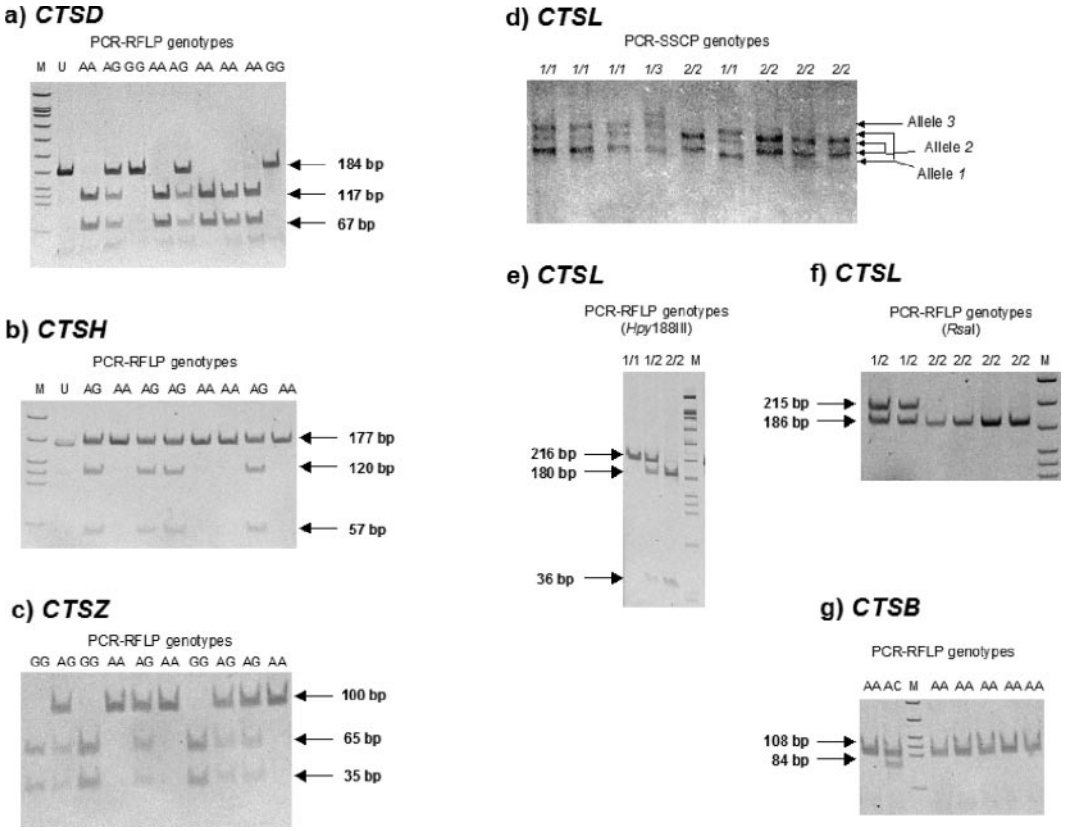

Figure 1. Polymerase chain reaction-RFLP and PCR-single strand conformation polymorphism (SSCP) genotypes. The genotypes are indicated at the top of each gel lane. a) PCR-RFLP at the cathepsin D (*CTSD*) locus; allele g.70A = A, allele g.70G = G. b) PCR-RFLP at the cathepsin H (*CTSH*) locus; allele g.122A = A, allele g.122G = G. c) PCR-RFLP at the cathepsin Z (*CTSZ*) locus; allele g.37A = A, allele g.37G = G. d) PCR-SSCP at the cathepsin L (*CTSL*) locus; allele 1 = g.[32C;34C], allele 2 = g.[32C;34T], allele 3 = and g.[32T;34T]. e) PCR-RFLP (*Hpy*188III) at the *CTSL* locus; allele 1 = g.[32C;34C] or g.[32T;34T], allele 2 = g.[32C;34T]. f) PCR-RFLP (*Rsa*I) at the *CTSL* locus; allele 1 = g.[32C;34C] or g.[32C;34T], allele 2 = g.[32T;34T]; the fragment of 29 bp is not shown in the gel. g) PCR-RFLP at the cathepsin B (*CTSB*) locus; allele g.72A = A, allele g.72C = C; the fragments of 31 and 24 bp are not shown in the gel. M = molecular DNA weight marker VIII (Roche Diagnostics, Basel, Switzerland); U = undigested PCR product. The PCR-RFLP patterns of the cystatin B (*CSTB*) and cathepsin F (*CTSF*) loci have been already reported in Russo et al. (2002, 2004), respectively.

cus (Figure 1g; g.72A, alleles 1 and 2; EMBL accession numbers AJ315558 and AJ315559; Russo et al., 2002). Thus, frequencies obtained for the 2 alleles having the g.72A variants were combined using the data reported by Russo et al. (2002).

In Silico Functional Prediction of Missense Mutations in CSTB, CTSF, and CTSZ Genes

The analyzed mutations in 3 investigated genes (*CSTB*, *CTSF*, and *CTSZ*) are missense mutations. Two of them have been already analyzed in other studies (*CSTB*, D63N: Russo et al., 2002; *CTSF*, D355E: Russo et al., 2003, 2004). The *CTSZ* missense mutation (K65R) was identified by in silico analysis and then experimentally confirmed in this study. Functional prediction of the consequences of AA change in the 3 proteins based on PANTHER gave subPSEC scores of −1.83702 ($P_{deleterious}$ = 0.23813), −3.11681 ($P_{deleterious}$ = 0.52917), and −4.02067 ($P_{deleterious}$ = 0.52917) for *CSTB*, *CTSF*, and *CTSZ*, respectively. According to these values, the *CSTB* mutation may have no functional effect, whereas the mutations in *CTSF* and *CTSZ* might have functional consequences.

Linkage Mapping of CTSD and CTSH

Because the *CTSD* gene has not yet been mapped in pigs, we used the detected polymorphism to genetically localize this locus. Twopoint sex-averaged analysis revealed that the *CTSD* gene (97 informative meiosis) is linked with loci already assigned to SSC2: *MUC5A5* [θ = 0.00; logarithm of odds (**LOD**) = 15.35], *Sw256* (θ = 0.16; LOD = 6.42), *TNNT3* (θ = 0.07; LOD = 6.15), and *CTSF* (θ = 0.07; LOD = 4.97). The multipoint sex-averaged map of SSC2 placed *CTSD* between *TNNT3* and *CTSF* (*TNNT3* − 5.9 cM − *MUC5A5* − 0.0 cM − *CTSD* − 13.1 cM − *CTSF* − 13.8 cM − *PYGM* − 0.0 cM − *Sw256* − 4.5 cM − *S0141* − 5.3

cM − *S0110* − 19.0 cM − *Sw240* − 13.9 cM − *FSHB* − 7.8 cM − *LDHA* − 0.0 cM − *S0170* − 8.3 cM − *Sw395* − 0.0 cM − *S0091* − 0.0 cM − *Sw776* − 10.7 cM − *INSR* − 0.0 cM − *S0226* − 0.0 cM − *Sw14* − 29.7 cM − *S0010*). This assignment confirms comparative mapping data between human chromosome 11 (**HSA**), which contains the *CTSD* gene (1.73 M, 11p15.5), and the distal p arm end of SSC2 in which occurred a small rearrangement that joined HSA11q13 loci (the human *CTSF* gene maps at 11q13, 66.09 M) with HSAp15 loci (Rink et al., 2006).

Twopoint sex-averaged linkage analysis was conducted also for the *CTSH* gene (34 informative meiosis) to confirm the physical assignment already obtained to SSC7 (Fontanesi et al., 2001b). As expected, several loci localized on this chromosome showed a LOD score >3: *CTSH*: *S0013* (θ = 0.03; LOD = 7.70), *S0029* (θ = 0.09; LOD = 5.57), *S0047* (θ = 0.03; LOD = 7.70), *S0066* (θ = 0.04; LOD = 6.27), *S0078* (θ = 0.06; LOD = 3.19), *S0102* (θ = 0.08; LOD = 4.50), *S0115* (θ = 0.16; LOD = 3.61), *SLA* (θ = 0.00; LOD = 6.02), and *TNFB* (θ = 0.03; LOD = 8.28).

Association Analysis Between DNA Markers and Production Traits

Association analysis was carried out in Italian Large White sib-tested pigs. All pigs but one carried the CC genotype at the *RYR1* g.1843C>T polymorphic site. The animal with genotype CT at this locus was not included in the following statistical evaluations. At the *CTSL* locus, only 2 pigs were heterozygous, whereas all others were homozygous. Therefore, this gene was not considered in the association analysis.

Animals homozygous for 1 of the 2 SNP alleles were very rare at the *CSTB* (g173AA, n. 1), *CTSB* (g.72CC, n. 1), *CTSD* (g.70GG, n. 4), and *CTSH* (g.122GG, n. 6) loci; thus, in these cases, association analyses were carried out merging the less frequent genotype class with the heterozygous class. Results of the association analysis for these loci are reported in Table 4. Almost overlapping results were obtained excluding from the association tests the animals with the less frequent genotype (data not shown). Table 5 reports the results for the *CTSF* and *CTSZ* loci that were obtained considering all 3 genotype classes. Only for these 2 loci was it possible to estimate additive and dominance effects (Table 6).

Using single marker-trait tests, the *P*-nominal value of 0.029 corresponded to a PFP threshold of 0.10. Hence, all single marker-trait tests were considered significant when $P \leq 0.029$ (underlined in Tables 4 and 5).

No analyzed markers of the cathepsin and cystatin loci were associated with CATB. No association was observed for the *CTSB* gene for which only 1 of the 2 previous studies reported a putative effect of allele g.72C on BFT (Russo et al., 2002, 2003).

The *CTSD* gene was highly significantly associated with all EBV. The rarer genotype (g.70AG) was associated with less ADG ($P < 0.0001$), less LC ($P < 0.0001$), less HW ($P < 0.001$), less FGR ($P < 0.001$), and greater BFT ($P < 0.001$). No meat quality traits were associated with the *CTSD* analyzed DNA marker.

The *CTSF* gene was associated with ADG ($P = 0.008$), LC ($P = 0.001$), and BFT ($P = 0.02$) for which genotype g.22CC increased growth performance and lean meat content and decreased the fatness trait. These results confirmed what was described previously for these traits (Russo et al., 2003). No significant result was obtained for FGR for which the previous investigation showed association with *CTSF* genotypes (Russo et al., 2003).

The *CTSH* gene was associated with FGR ($P = 0.028$). Average daily gain was close to the 0.10 PFP threshold. In this case, the most frequent genotype (g.122AA) was associated with less FGR and less ADG.

The *CTSZ* gene was associated with ADG ($P = 0.006$), LC ($P = 0.01$), HW ($P = 0.024$), and FGR ($P = 0.029$) with favorable additive genetic effects of allele g.37G on EBV (Table 6). Glycogen content was close to the 0.10 PFP threshold.

No significant association was obtained for the *CSTB* gene even though for ADG a putative effect (*P*-nominal value = 0.099) of the animals with the rarer genotype (g.173AG) on less ADG was in the same direction as that was already observed in 2 different studies (Russo et al., 2002, 2003).

Haplotypes between the 2 cathepsin loci (*CTSD* and *CTSF*) that mapped a few centimorgans from each other on SSC2 were inferred for the second group of sib-tested Italian Large White pigs. The 4 possible haplotypes (*CTSD* g.70A, *CTSF* g.22C = haplotype 1; *CTSD* g.70G, *CTSF* g.22G = haplotype 2; *CTSD* g.70A, *CTSF* g.22G = haplotype 3; *CTSD* g.70G, *CTSF* g.22C = haplotype 4) showed the following frequencies: 1 = 0.707; 2 = 0.046; 3 = 0.212; 4 = 0.035. These haplotype frequencies depend largely on the allele frequencies of these 2 markers, because linkage disequilibrium in this population was low ($D' = 0.277$, SD = 0.097). Association analysis considering the genotype classes constituted by 0, 1, and 2 copies of haplotype 1 confirmed the *CTSD* and *CTSF* single marker test effects (Table 7). Animals with 2 copies of haplotype 1 showed greater ADG, LC, and HW ($P = 0.011$, 0.008, and 0.021, respectively) and less BFT and FGR ($P = 0.092$ and 0.079, respectively). Suggestive association was observed also between muscle lactate content and these haplotype classes ($P = 0.074$). Association analysis considering the genotype classes constituted by 0, 1, and 2 copies of haplotype 3 showed significant results only for muscle glycogen and lactate content ($P = 0.025$ and 0.041, respectively; Table 7). The class with 2 copies of haplotype 3 showed less glycogen and greater lactate content and, probably for these opposite effects, GP was not influenced.

Table 4. Association analysis between cathepsin B (*CTSB*), cathepsin D (*CTSD*), cathepsin H (*CTSH*), and cystatin B (*CSTB*) markers and meat quality variables and EBV[1]

Genes[2]/traits[3]	LSM (SE)	Number of animals	LSM (SE)	Number of animals	*P*-value[4]
CTSB	g.72AA		g.72AC + g.72CC		
pH_l	5.936 (0.017)	238	5.890 (0.050)	32 + 1	0.349
pH_u	5.664 (0.016)	238	5.641 (0.041)	32 + 1	0.604
Glycogen	48.606 (1.880)	238	46.692 (4.606)	32 + 1	0.688
Lactate	56.781 (1.072)	238	57.301 (3.152)	32 + 1	0.876
GP	105.560 (1.857)	238	102.490 (4.659)	32 + 1	0.527
CATB	1.634 (0.021)	404	1.664 (0.044)	49 + 1	0.461
EBV ADG	+32.986 (1.391)	404	+29.873 (3.916)	49 + 1	0.454
EBV LC	+1.887 (0.094)	404	+1.913 (0.265)	49 + 1	0.927
EBV BFT	−2.331 (0.194)	404	−2.247 (0.546)	49 + 1	0.885
EBV HW	+0.585 (0.039)	238	+0.689 (0.120)	32 + 1	0.421
EBV FGR	−0.151 (0.010)	238	−0.138 (0.031)	32 + 1	0.685
CTSD	g.70GG + g.70AG		g.70AA		
pH_l	5.891 (0.039)	4 + 36	5.934 (0.018)	231	0.297
pH_u	5.672 (0.034)	4 + 36	5.656 (0.016)	231	0.642
Glycogen	43.508 (3.694)	4 + 36	49.447 (1.870)	231	0.128
Lactate	59.674 (2.478)	4 + 36	56.296 (1.065)	231	0.204
GP	103.770 (3.747)	4 + 36	105.530 (1.857)	231	0.656
CATB	1.147 (0.038)	4 + 36	1.160 (0.018)	231	0.739
EBV ADG	+16.825 (4.070)	4 + 36	+36.169 (1.693)	231	<u>≤0.0001</u>
EBV LC	+0.869 (0.283)	4 + 36	+2.188 (0.118)	231	<u>≤0.0001</u>
EBV BFT	−0.220 (0.588)	4 + 36	−2.394 (0.2447)	231	<u>0.0007</u>
EBV HW	+0.256 (0.094)	4 + 36	+0.639 (0.039)	231	<u>0.0002</u>
EBV FGR	−0.064 (0.024)	4 + 36	−0.163 (0.010)	231	<u>0.0001</u>
CTSH	g.122AA		g.122AG + g.122GG		
pH_l	5.918 (0.019)	192	5.950 (0.028)	70 + 6	0.335
pH_u	5.653 (0.017)	192	5.672 (0.024)	70 + 6	0.497
Glycogen	47.849 (1.956)	192	49.831 (2.707)	70 + 6	0.504
Lactate	57.541 (1.166)	192	54.924 (1.785)	70 + 6	0.213
GP	105.430 (1.969)	192	104.830 (2.744)	70 + 6	0.843
CATB	1.162 (0.020)	192	1.149 (0.027)	70 + 6	0.673
EBV ADG	+31.139 (1.897)	192	+38.792 (3.010)	70 + 6	0.032
EBV LC	+1.914 (0.133)	192	+2.194 (0.210)	70 + 6	0.260
EBV BFT	−1.837 (0.271)	192	−2.668 (0.431)	70 + 6	0.104
EBV HW	+0.558 (0.0437)	192	+0.645 (0.069)	70 + 6	0.289
EBV FGR	−0.136 (0.011)	192	−0.181 (0.0174)	70 + 6	<u>0.028</u>
CSTB	g.173AA + g.173AG		g.173GG		
pH_l	6.001 (0.070)	1 + 11	5.924 (0.017)	247	0.284
pH_u	5.688 (0.060)	1 + 11	5.657 (0.016)	247	0.610
Glycogen	44.337 (6.515)	1 + 11	48.705 (1.789)	247	0.513
Lactate	59.338 (4.512)	1 + 11	56.652 (1.020)	247	0.562
GP	103.340 (6.625)	1 + 11	105.360 (1.784)	247	0.767
CATB	1.610 (0.062)	1 + 23	1.636 (0.020)	457	0.674
EBV ADG	+22.750 (5.685)	1 + 23	+32.367 (1.303)	457	0.099
EBV LC	+1.289 (0.387)	1 + 23	+1.885 (0.089)	457	0.134
EBV BFT	−1.100 (0.797)	1 + 23	−2.349 (0.183)	457	0.127
EBV HW	+0.581 (0.176)	1 + 11	+0.582 (0.038)	247	0.995
EBV FGR	−0.108 (0.044)	1 + 11	−0.150 (0.010)	247	0.346

[1]Least squares means (LSM) are reported with their SE (in parentheses) for each genotypic class. The number of animals for each genotype class is reported.

[2]Genotyped pigs at the *CTSB* and *CSTB* loci reported by Russo et al. (2003; first group of sib-tested Italian Large White pigs: n = 210), for which data for CATB and 3 EBV (ADG, LC, and BFT) were available, were combined with genotyped pigs of the second group of sib-tested Italian Large White pigs (272 animals). One pig was excluded from the association analysis because it had genotype CT at the ryanodine receptor 1 g.1843C>T polymorphic site. Not all animals of the first group were genotyped for the *CTSB* marker. The less frequent genotypic class was merged with the heterozygous class.

[3]pH_l = pH measured at 2 h postmortem on musculus semimembranosus; pH_u = pH measured at 24 h postmortem on the same muscle; GP = glycolytic potential; CATB = cathepsin B activity; LC = lean cuts; BFT = backfat thickness; HW = ham weight; FGR = feed:gain ratio.

[4]*P*-nominal values corresponding to proportion of false positives ≤0.10 are underlined.

Table 5. Association analysis between cathepsin F (*CTSF*) and cathepsin Z (*CTSZ*) markers and meat quality variables and EBV[1]

Genes[2]/ traits[3]	LSM (SE)	Number of animals	LSM (SE)	Number of animals	LSM (SE)	Number of animals	*P*-value[4]
CTSF	g.22GG		g.22CG		g.22CC		
pH_l	5.984 (0.050)	25	5.930 (0.027)	90	5.918 (0.021)	156	0.467
pH_u	5.668 (0.042)	25	5.654 (0.023)	90	5.660 (0.019)	156	0.948
Glycogen	49.946 (4.624)	25	49.885 (2.581)	90	47.415 (2.121)	156	0.671
Lactate	50.959 (3.124)	25	58.279 (1.636)	90	56.763 (1.280)	156	0.112
GP	100.030 (4.663)	25	107.980 (2.570)	90	104.420 (2.096)	156	0.226
CATB	1.634 (0.044)	52	1.630 (0.025)	190	1.639 (0.025)	239	0.952
EBV ADG	+27.173 (3.837)	52	+28.221 (2.007)	190	+35.811 (1.790)	239	0.008
EBV LC	+1.622 (0.260)	52	+1.519 (0.1360)	190	+2.175 (0.121)	239	0.001
EBV BFT	−2.198 (0.539)	52	−1.712 (0.282)	190	−2.773 (0.251)	239	0.020
EBV HW	+0.665 (0.121)	25	+0.473 (0.0639)	90	+0.632 (0.049)	156	0.109
EBV FGR	−0.1355 (0.031)	25	−0.139 (0.016)	90	−0.156 (0.012)	156	0.640
CTSZ	g.37AA		g.37AG		g.37GG		
pH_l	5.896 (0.032)	63	5.952 (0.024)	116	5.919 (0.027)	92	0.297
pH_u	5.677 (0.027)	63	5.665 (0.021)	116	5.637 (0.023)	92	0.435
Glycogen	42.471 (2.971)	63	49.578 (2.272)	116	50.980 (2.578)	92	0.046
Lactate	59.118 (1.973)	63	55.182 (1.473)	116	57.219 (1.647)	92	0.254
GP	100.940 (3.027)	63	104.920 (2.311)	116	108.670 (2.622)	92	0.112
CATB	1.124 (0.031)	63	1.153 (0.023)	116	1.188 (0.027)	92	0.231
EBV ADG	+25.746 (3.299)	63	+32.526 (2.431)	116	+39.489 (2.730)	92	0.006
EBV LC	+1.624 (0.230)	63	+1.830 (0.169)	116	+2.452 (0.190)	92	0.010
EBV BFT	−1.344 (0.476)	63	−2.165 (0.351)	116	−2.456 (0.394)	92	0.189
EBV HW	+0.482 (0.076)	63	+0.527 (0.056)	116	+0.720 (0.063)	92	0.024
EBV FGR	−0.105 (0.019)	63	−0.154 (0.014)	116	−0.171 (0.016)	92	0.029

[1]Least squares means (LSM) are reported with their SE (in parentheses) for each genotypic class. The number of animals for each genotype class is indicated.

[2]Genotyped pigs at the *CTSF* locus reported by Russo et al. (2003; first group of sib-tested Italian Large White pigs: n = 210), for which data for CATB and 3 EBV (ADG, LC, and BFT) were available, were combined with genotyped pigs of the second group of sib-tested Italian Large White pigs (272 animals). One pig was excluded from the association analysis because it had genotype CT at the ryanodine receptor 1 g.1843C>T polymorphic site.

[3]pH_l = pH measured at 2 h postmortem on musculus semimembranosus; pH_u = pH measured at 24 h postmortem on the same muscle; GP = glycolytic potential; CATB = cathepsin B activity; LC = lean cuts; BFT = backfat thickness; HW = ham weight; FGR = feed:gain ratio.

[4]*P*-nominal values corresponding to proportion of false positives ≤0.10 are underlined.

DISCUSSION

Cathepsins are lysosomal proteinases with a broad spectrum of functions exerted in most, if not in all, tissues and cell types (Brix et al., 2008). These enzymes, usually synthesized as preprocathepsins, ensure turnover of metabolites and cell and tissue structures via bulk protein degradation but also possess highly specific and directed proteolytic activities. They, for example, participate in antigen processing and presentation, with effects on immune response (Zavašnik-Bergant and Turk, 2006), hormone and proenzyme processing with

Table 6. Additive and dominance effects (with SE in parentheses) obtained for the cathepsin F (*CTSF*) and cathepsin Z (*CTSZ*) marker tests[1]

Genes/traits[2]	Additive effect (SE)	*P*	Dominance effect (SE)	*P*
CTSF				
EBV ADG	4.319 (2.1179)	0.042	−3.271 (2.918)	0.263
EBV LC	0.277 (0.143)	0.054	−0.379 (0.198)	0.056
EBV BFT	−0.288 (0.297)	0.334	0.773 (0.410)	0.060
CTSZ				
Glycogen	4.255 (1.80)	0.019	2.852 (2.711)	0.294
EBV ADG	6.871 (2.141)	0.001	−0.092 (3.240)	0.977
EBV LC	0.414 (0.149)	0.006	−0.208 (0.225)	0.356
EBV HW	0.119 (0.049)	0.016	−0.074 (0.075)	0.324
EBV FGR	−0.033 (0.012)	0.009	−0.016 (0.019)	0.383

[1]Results are reported only for association analyses with $P < 0.10$.

[2]The number of animals was reported in Table 5. LC = lean cuts; BFT = backfat thickness; HW = ham weight; FGR = feed:gain ratio.

Table 7. Association analysis between the cathepsin D (*CTSD*) and cathepsin F (*CTSF*) haplotypes and meat quality variables and EBV[1]

	Haplotype classes[2]			
Haplotypes/traits[2]	0	1	2	*P*
1 vs. 2, 3, and 4				
Lactate	47.318 (4.152)	57.423 (1.988)	57.566 (1.886)	0.074
EBV ADG	+28.074 (5.051)	+28.409 (2.561)	+38.036 (2.226)	0.011
EBV LC	+1.516 (0.350)	+1.675 (0.178)	+2.327 (0.154)	0.008
EBV BFT	−1.389 (0.726)	−1.606 (0.368)	−2.559 (0.320)	0.092
EBV HW	+0.616 (0.116)	+0.455 (0.059)	+0.672 (0.051)	0.021
EBV FGR	−0.127 (0.029)	−0.127 (0.015)	−0.169 (0.013)	0.079
3 vs. 1, 2, and 4				
Glycogen	59.532 (6.221)	53.572 (3.242)	45.480 (2.156)	0.025
Lactate	45.382 (4.580)	55.794 (2.387)	57.868 (1.588)	0.041

[1]Least squares means are reported with their SE (in parentheses). The number of animals for each haplotype class is the following: analysis 1 vs. 2, 3, and 4, class 0 = 27 pigs, class 1 = 105 pigs, class 2 = 139 animals. Analysis 3 vs, 1, 2, and 4, class 0 = 177 pigs, class 1 = 73 pigs, class 2 = 21 animals. Only results with $P < 0.10$ are reported.

[2]Haplotype classes have 0, 1, or 2 copies of haplotype 1 (1 vs. 2, 3, and 4) or 3 (3 vs. 1, 2, and 4). LC = lean cuts; BFT = backfat thickness; HW = ham weight; FGR = feed:gain ratio.

effects on biochemical pathway regulation, and activation (Dunn et al., 1991; Brix et al., 2001; Barros et al., 2004; Hook et al., 2004). Moreover, they are involved in cancer progression and metastasis, as well as cell death through mediation of apoptosis (Mohamed and Sloane, 2006; Stoka et al., 2007). High cathepsin activities of porcine skeletal muscle have been correlated to defects of dry-cured hams associated with excessive meat softness together with stickiness on chewing, dark color, astringent or metallic aftertastes, depots of tyrosine crystals, and formation of white films on the cut surface (Virgili et al. 1995a,b, 1998) accounting for about 1 to 3% of discarded legs in the production of high-quality protected denomination of origin hams, which are a very important source of income for the pork industry in countries such as Italy, Spain, France, and Slovenia (Russo and Nanni Costa, 1995; Virgili and Schivazappa, 2002; Bosi and Russo, 2004).

Cathepsin B, CTSF, CTSH, CTSL, and CTSZ are cysteine lysosomal proteinases with endopeptidase, peptidyl-dipeptidase, aminopeptidase, or carboxypeptidase activities and with expression in a wide range of tissues (CTSB, CTSH, CTSL, and CTSZ) or mainly expressed in striated muscles (CTSF). Cathepsin D is a ubiquitous aspartic lysosomal proteinase with endopeptidase activity (Barrett et al., 1998). Cathepsins, which are generally active in the acidic lysosomal environment, have as intracellular (type 1) or extracellular (type 2) inhibitors several small proteins known as cystatins. Cystatin B is a type 1 cystatin found to be widely distributed among different cells and tissues. It was originally discovered as an inhibitor of CTSB and later recognized to have greater affinity for CTSH and CTSL (Lenney et al., 1979; Turk and Bode, 1991; Lenarčič et al., 1996; Abrahamson et al., 2003).

Considering the effects of cathepsin activity in determining sensory and texture quality of dry-cured hams, the genes encoding for these lysosomal enzymes and their inhibitors can be considered candidates that should be investigated to identify DNA markers associated with these dry-cured ham variables. On the other hand, due to the important biological roles that these enzymes play in a large number of key physiological functions, cathepsin and cystatin genes may be candidates for several other economically important traits in pigs, such as performance, carcass, and meat production traits.

In silico mining of the EST database made it possible to identify several putative SNP in porcine cathepsin genes. Among them, a few, for which it was possible to design simple PCR-RFLP tests, were chosen for validation in the Italian Large White pig population to obtain useful DNA markers for association studies. In this way, DNA markers were identified for porcine *CTSD*, *CTSH*, and *CTSZ* genes confirming the utility of exploiting EST sequences to detect SNP segregating in commercial pig populations (Hayes et al., 2007). New polymorphisms were in vitro-identified in the porcine *CTSL* gene, but the low level of heterozygosity excluded the possibility of using the markers at this locus in the sib-tested pig population and for linkage mapping. However, this locus was more informative in the Duroc breed for which 3 alleles were observed. The Duroc breed was very different from the other breeds also at the *CTSZ* locus for which allele g.37G was almost fixed. Allele frequency indications at the investigated loci will be useful to select informative markers for association studies.

All analyzed genes, except *CTSD*, were previously mapped in the pig genome. We linkage-mapped *CTSD* on the p telomeric end of SSC2 in an important complex QTL region for feed efficiency, growth, and meat quality and carcass traits with or without parent-of-origin effects (de Koning et al., 1999; Jeon et al., 1999; Nezer et al., 1999; Bidanel et al., 2001; Lee et al., 2003; Houston et al., 2005; Stearns et al., 2005; Sanchez et

al., 2006). The *IGF2* intron3-g.3072G>A polymorphic site has been proposed to be the causative mutation for the imprinted QTL effects identified in this region, even if the presence of additional mutations have been supposed (Van Laere et al., 2003; Jungerius et al., 2004; Sanchez et al., 2006). The *CTSD* gene resulted close to *CTSF*, which was previously genetically mapped by Russo et al. (2004). The results of the association study in the Italian Large White population indicated that *CTSD* is associated with all EBV considered (ADG, LC, BFT, HW, and FCR) and *CTSF* with 3 of them (ADG, LC, and BFT). These results were confirmed using haplotype information of the SSC2p end obtained combining *CTSD* and *CTSF* genotypes. The results observed with the 2 cathepsin genes could be due to a positional effect. However, because linkage disequilibrium in the Italian Large White population seems low (Davoli et al., 2006), it is tempting to hypothesize a direct functional role of these 2 genes (or a very close gene) in the observed effects. To further suggest a functional role of the identified mutations, the missense substitution identified in the *CTSF* gene is in a highly conserved protein region where all mammals carried D at position 355 (Russo et al., 2004). This AA change might have a functional effect as suggested by PANTHER in silico prediction. Moreover, according to the results of our previous investigation (Russo et al., 2003) and those recently reported by Ramos et al. (2008), here we confirmed the effects observed for *CTSF* on most performance and carcass traits. Investigation of these 2 cathepsin genes together with the *IGF2* intron3-g.3072G>A substitution in a specific designed experiment that should be able to give indications on parent of origin effects might provide evidence supporting or excluding direct involvement of the cathepsin genes in affecting the traits for which QTL segregate in this region of SSC2.

Among the other investigated genes, *CSTB* was previously localized at the telomeric end of SSC13q (Russo et al., 2002). Single marker analysis revealed association between ADG and a microsatellite (*S0291*) mapped in the same chromosome region in a Duroc × Yorkshire F2 population (Kim et al., 2006). Two previously reported studies (Russo et al., 2002, 2003) indicated an effect of this polymorphism on ADG with the rarer allele associated with less ADG. Although in the present study association between ADG and the *CSTB* marker was not significant at the 0.10 PFP level, it was in the same direction. This allele is the only one identified in the Meishan breed, which notoriously has a decreased growth rate compared with Euro-American breeds.

Cathepsin B was placed on SSC14 (Russo et al., 2002) in a region where suggestive QTL for fat deposition and lean meat content have been identified (Rohrer and Keele, 1998a; Dragos-Wendrich et al., 2003), but in our study, we did not confirm the association with BFT observed by Russo et al. (2002). A subsequent analysis of this locus (Russo et al., 2003) did not confirm the previous result. Differences between the former study and that reported in Russo et al. (2003) and in this investigation could be the origin of this discrepancy. In Russo et al. (2002), a composite population was used for the association study (Italian Large White, Italian Landrace, and Italian Duroc animals), and the trait considered was a phenotypic measure and not an EBV. Moreover, additional alleles were utilized in the association study, even if the same genotype information was used in Russo et al. (2003).

Cathepsin H was already mapped to SSC7 by somatic cell hybrid panel analyses (Fontanesi et al., 2001b). Using the identified *CTSH* DNA marker, we confirmed this assignment by linkage analysis even if a multipoint map including *CTSH* was not possible to build with high confidence due to the low number of informative meiosis for this gene. However, according to the two-point linkage data and human porcine comparative mapping information (Rink et al., 2006; human *CTSH* gene is localized on HSA15q24-q25), the porcine *CTSH* gene should be more precisely localized on SSC7q very close to the *SLA* region, where a large number of reports indicated the presence of several important QTL for backfat, growth, and meat content (i.e., Rothschild et al., 1995; Rohrer and Keele, 1998a,b; Bidanel et al., 2001; Malek et al., 2001b; Paszek et al., 2001; Nezer et al., 2002; Ovilo et al., 2002; Reiner et al., 2002; Yue et al., 2003; Rohrer et al., 2005; Gilbert et al., 2007). The identified *CTSH* marker was not very informative in the sib-tested pig population, but association analysis was significant for FGR and close to the significance PFP threshold for ADG, 2 correlated traits for which the obtained results could be due to a positional effect even if direct involvement of this gene cannot be ruled out. Interestingly, the allele at less frequency seems to have favorable effects on growth and feed efficiency. This is unexpected, because selection toward growth efficiency currently under way in this population for many years might have increased the frequency of positive alleles as evidenced also for *CTSD*, *CTSF*, and *CSTB*. It could be possible that the less frequent allele is associated with unfavorable effects on other traits. However, the complex QTL pattern of this region (Gilbert et al., 2007), in which cryptic QTL alleles were also identified (de Koning et al., 1999; Yue et al., 2003), might be the cause of our observations for the *CTSH* marker.

The *CTSL* gene was previously mapped to SSC10 (Fontanesi et al., 2001b) in a region where QTL for fat deposition and growth traits have been mapped (Knott et al., 1998; Rohrer et al., 2005). The SNP identified in this gene were not useful in the association study conducted in our population.

Finally, *CTSZ* was previously linkage-mapped to SSC17 at about 110 cM (Ramos et al., 2006). Several QTL for growth, meat quality, and carcass traits have been mapped on this chromosome (Malek et al., 2001a; Pierzchala et al., 2003; Thomsen et al., 2004). In our population, *CTSZ* was associated with ADG, LC, HW, and FGR. Thomsen et al. (2004) showed the presence of a QTL with parent of origin effects for early growth

rate around 80 to 90 cM, and Pierzchala et al. (2003) obtained indications of the presence of QTL for several fat deposition traits in a large interval on SSC17. Backfat thickness, even if not significant in the association study with *CTSZ*, showed genotype least squares means that were in the same direction as would be expected due to the negative correlation with ADG. In silico functional prediction of the *CTSZ* K65R mutation indicated that this substitution might have functional consequences. This could support a direct role of this mutation in determining the identified effects on production traits.

Analyzed markers did not show any association with CATB, confirming for *CSTB*, *CTSB*, and *CTSF* what was previously reported by Russo et al. (2002, 2003). Thus, it could be possible that genes other than these investigated candidate genes affect this trait. Further studies will be carried out to analyze other cathepsin and cystatin genes to identify DNA markers associated with this trait, which, in turn, is correlated with the occurrence of defects of the hams during the curing process (Virgili et al., 1995b, 1998; Garcia-Garrido et al., 2000).

Several markers in cathepsin genes (*CTSD*, *CTSF*, *CTSH*, and *CTSZ*) have been shown to be associated with growth, fat deposition, and production traits in the Italian Large White pig population. Results for *CTSF* are consistent with previous investigations conducted on other groups of pigs. Further investigation will be carried out to verify the results obtained for *CTSD*, *CTSH*, and *CTSZ*. However, the biochemical and physiological functions of the lysosomal proteinases, together with the results obtained in our investigation, might suggest that the cathepsin gene family plays important roles in affecting economically important traits in pigs. To evaluate this hypothesis, other cathepsin genes should be investigated and additional association studies with production traits should be conducted in Italian Large White pigs, as well as in other pig populations. Cathepsin D seemed the most interesting gene, because the tested marker gave the most significant results. Because the *CTSD* gene maps to SSC2 close to the *IGF2* gene in the parent of origin QTL region, linkage disequilibrium between this marker and the *IGF2* intron3-g.3072G>A mutation should be evaluated before considering the *CTSD* polymorphism in marker-assisted selection programs. It will also be of interest to determine if imprinting effects control the expression of *CTSD* as already reported for *IGF2* and to evaluate if the identified marker has functional significance.

Moreover, the SNP investigated in the cathepsin and cystatin genes were not associated with CATB and did not have large impacts on the other investigated meat quality traits. For all meat quality traits, but in particular for CATB, whose related defect is evidenced late in the heavy pigs production chain, marker-assisted selection would be highly beneficial to improve selection efficiency. Thus, additional studies are required to identify genes affecting CATB, for which a high concentration has an important negative impact on quality of dry-cured hams.

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