animal

Histological characteristics of *longissimus dorsi* muscle and their correlation with restriction fragment polymorphisms of calpastatin gene in F_2 Jinghua $\times$ Piétrain crossbred pigs

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*In order to evaluate the genotype of the calpastatin (*CAST*) gene and its relationship to muscle histology and other* post mortem *traits in the Jinghua $\times$ Piétrain F_2 pig family, 158 barrows and gilts were electrically stunned and exsanguinated. Both blood and muscle samples were collected, and both* post mortem *traits and meat qualities were recorded. Restriction fragment length polymorphism (RFLP) analysis, the periodic acid Schiff reaction (PAS) and myosin heavy-chain immunohistochemistry were employed to explore the relationship between genotype and muscle histology. Based on PAS reactivity, muscle fibres can be classified into three types: PAS ($-$), PAS ($+$) and PAS ($++$). Myosin heavy-chain immunohistochemistry can differentiate muscle fibres into either slow or fast fibres; the proportion of slow and fast fibres were 6% and 94%, respectively. When the amplification products of the* CAST *gene were digested with* Msp*I,* Hinf*I and* Rsa*I, two different cleavage patterns could be discriminated from the endonuclease map detected using each enzyme. The results showed that the polymorphisms detected using these three endonucleases are identical. Only three genotypes (AA/CC/EE, AB/CD/EF and BB/DD/FF) were distinguished. Their frequencies were 0.1835, 0.5823 and 0.2342, respectively. Different genotypes had significant association with area and* pH_{45m} *value of loin muscle, while showing no significant association with the water-holding capacity and conductivity of loin muscle. The results also revealed that the genotypes had a significant correlation with diameter, area, circularity and the aspect ratio of muscle fibres. It was also presented that the genotypes significantly correlated with the percentage of intramuscular connective tissue.*

Introduction

Pork quality varies a great deal both within and between animals with different genetic and environmental backgrounds. Many characteristics of muscle tissue such as density, diameter, cross-sectional area, proportion of red fibres, and glycogen content directly affect meat quality (Karlsson *et al.*, 1999).

Calpastatin (CAST) is a natural and specific inhibitor of calpain, and its activity is related to the amount of inhibitor protein (Pontremoli *et al.*, 1988). Earlier studies have shown a correlation between calpain activity and fibre necrosis in dystrophic muscle. Transgenic mice that overexpressed calpastatin in muscle had limited muscle necrosis (Spencer and Mellgren, 2002). This corresponding gene was mapped

on chromosome 2 in pigs (Rettenberger *et al.*, 1996). It is well documented that calpastatin activity after death is closely related to meat tenderness (Parr *et al.*, 1999). Calpastatin gene RFLP genotypes show a marked effect on some meat quality characteristics. Although some indicative associations were reported in cattle (Chung *et al.*, 2001) and pigs (Koćwin-Podsiadła *et al.*, 2003), the relationship between variation in sequence or changes in *CAST* gene and phenotypic differences in meat quality is still not clear. There are polymorphic *Msp*I, *Hinf*I and *Rsa*I restriction sites in the *CAST* gene amplification products of pigs (Ernst *et al.*, 1998). The frequency of its genotypes varies with the breed.

As mentioned above, there are some reports of how the *CAST* gene influences meat quality in general and meat tenderness in particular. The relationship between polymorphism of the *CAST* gene and histological characteristics is still not clear. The purpose of this paper is to evaluate

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some genotypes containing different forms of the *CAST* gene and their relationship with histological characteristics of muscle and a few other traits that affect meat quality.

Material and methods

Animals and treatments

A total of 158 barrows and gilts ((Jinghua × Piétrain)× (Jinghua × Piétrain), JP F$_2$) weighing 35 kg each on average were allotted randomly within sex and sire groups to 16 pens. When the pigs had attained the appropriate slaughter weight (87.53 ± 7.69 kg), they were electrically stunned and exsanguinated. Then the blood samples were collected and the carcasses were eviscerated according to standard commercial procedures. Hot carcass weights were recorded and used to calculate dressing percentage. At 45 min *post mortem*, carcass length (from the posterior edge of the symphysis pubis to the anterior edge of the first rib) was recorded, and then the carcasses were split down the centre of the vertebral column. A 2 cm × 1 cm × 1 cm clip of *longissimus dorsi* muscle (LM) section was excised on the centre region at the level between the last rib and the last second rib from left sides after carcass data were collected. The tissue clips were prefixed in 10% neutral buffer formalin for 18 to 24 h for further histological research. Furthermore, an incision was made into the LM muscle and initial muscle pH was measured with a pH meter equipped with a glass electrode. Water-holding capacity (WHC) was measured according to the Carver Press method. Loin muscles at the 10/11th rib of nearside carcass were outlined on cellophane papers. Then scan the outlines by a scanner (600 d.p.i.) and the areas of the loin muscles were analysed by ImageJ (NIH shareware; National. Institutes of Health, Bethesda, MA, USA).

Histological examination

The muscle samples of JP F$_2$ pigs were fixed in 10% neutral buffered formalin for another 18 h. The tissue samples were dehydrated with gradient alcohol, cleared with xylene and then were dropped on melted paraffin. After that, the tissues were embedded in paraffin blocks, and serially sectioned to produce 6 to 8-μm-thick sections. In one group, the sections were stained with haematoxylin and eosin (HE) and in the other with routine periodic acid Schiff (PAS) reaction protocol and counterstained with Mayer's haematoxylin.

Immunohistochemistry

Mouse anti-myosin heavy-chain antibodies NCL-MHCs and NCL-MHCf (Novocastra Inc., Newcastle, UK) were employed as the primary antibody. EDTA antigen retrieval solution was used for retrieving the antigen by heating at 98°C for 30 min in a water bath, and the solution was allowed to cool naturally. The sections were rinsed in PBS (phosphate buffer with salt) and incubated with 0.1% trypsin (Biobasic Inc., Markham, Ont., Canada) in PBS for 5 min at 37°C.

Endogenous peroxidase activity was quenched with 3% H$_2$O$_2$ in distilled water for 10 min at room temperature. After rinsing, the sections were incubated for 30 min at room temperature with 10% normal goat serum. Subsequently, they were incubated with a primary antibody (diluted 1 : 60 with distilled water) for 24 h at 4°C. PBS substituting for the primary antibody served as a negative control. The sections were then incubated with biotin-conjugated rabbit anti-mouse IgG reagent (Dingguo Biotechnology Inc., Beijing, China) for 60 min at 37°C. After rinsing in PBS, the sections were incubated in horseradish peroxidase-conjugated streptin (Dingguo Biotechnology Inc., Beijing, China) for 40 min at 37°C. After that, the sections were stained with diaminobenzidine (DAB) solution (Lab Vision, Fremont, CA, USA) and counterstained with Mayer's haematoxylin.

Preparation of genomic DNA for PCR

About 1 ml of blood sample was centrifuged at 2000 × **g** for 10 min. The pellet was resuspended and incubated in 500 μl of enzyme incubation buffer (20 mmol/l Tris–HCl (pH 8.0), 1.2% Triton and 2 mmol/l EDTA) for 30 min at 37°C. Then, 25 μl of proteinase K (20 mg/ml) was added and the suspension was incubated at 56°C for 2 h. The DNA was extracted with phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and precipitated with ethanol. The precipitate was dissolved in 200 μl TE (10 mmol/l Tris–HCl, 1 mmol/l EDTA, pH 8.0) and its concentration was determined by UV2000 (Amersham Pharmacia, Uppsala, Sweden).

Amplification of CAST gene by PCR

Based on prior studies (Takano *et al.*, 1988; Ernst *et al.*, 1998), the sense primer (5′-GCGTGCTCATAAAGAAAAAGC-3′) and antisense primer (5′-TGCTACACC AGTAACAG-3′) were used to amplify a 1423-bp DNA sequence of the *CAST* gene. PCR reaction mixtures (50 μl each) were prepared by using 2.5 U of Taq DNA polymerase (Biobasic Inc., Markham, Ont., Canada), 6 μl of 25 mmol/l MgCl$_2$, 0.2 mmol/l of dNTP, 0.5 μmol/l of each primer and 5 μl template DNA. The reaction mixture was cycled 40 times in a GeneAmp System 2400 (Perkin Elmer): denaturing at 94°C for 30 s, annealing at 58°C for 45 s and extension at 72°C for 1 min. The PCR products were resolved by electrophoresis with 1.5% agarose and made visible by UV illumination (Jeda Company, Nanjing, China).

Restriction endonuclease digestion and polymorphism analysis

The amplification (10 μl) was digested for 5 h at 37°C in 20 μl of *Msp*I, *Hin*fI and *Rsa*I (Fermentas Inc., Burlington Ont., Canada). The digested products were electrophoresed in 2% agarose and photographed.

Statistical analyses

The polymorphisms of the *CAST* gene were assigned to the 158 JP F$_2$ pigs. The histological effects of the genotypes

were estimated using a GLM model (in which set the genotypes, sex and pen as fixed effects) (Statistical Analysis Systems Institute, 2001). The effect of each variable was tested by Turkey's multiple comparison with least-square means.

Results

Histochemical characteristic of the muscle fibre
PAS reaction

The muscle fibres are arranged in a very dense pattern (Figure 1). The fibres can be divided into two groups depending on their reaction to PAS. Type I fibres comprise PAS-negative fibres; the middle-sized fibres, round or ellipsoid, are distributed in clusters or widely dispersed. Type II fibres are PAS-positive and can be generally subdivided into two groups depending on the intensity of the reaction: a small group showing a strong PAS reaction and a large one showing moderately strong PAS reaction (Table 1).

The MHC immunohistochemistry of the longissimus
dorsi *muscle*

Mouse anti-MHCs or -MHCf monoclonal antibody was employed to type the muscle fibres. The muscle fibres were of two types (Figure 2), with MHC-positive fibres making up $6.05 \pm 1.84\%$ of the total. The muscle fibres can be classified into slow fibres and fast fibres by MHCf antibody immunohistochemistry, the latter accounting for approximately 94% of the fibres.
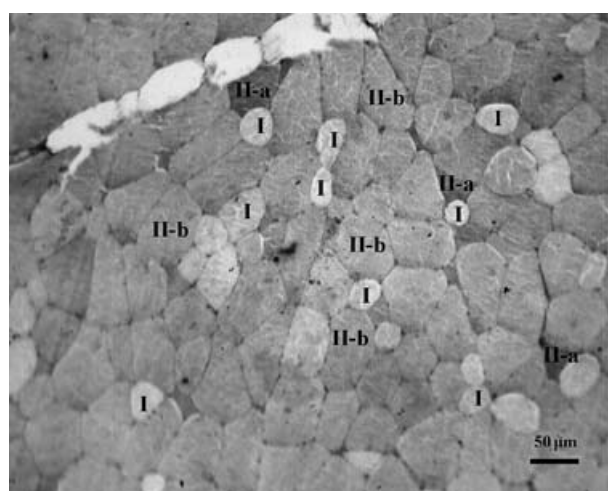
Polymorphism analysis of the CAST gene
Four fragments – 275, 370, 502 and 646 bp – were obtained when the amplification product was digested by

Table 1 *The periodic acid Schiff reaction (PAS) histochemical classification and comparison of muscle fibre in JP F_2 pigs (data are quoted as mean $\pm$ s.d.)*

PAS reaction	Percent (%)	Diameter (μm)	Optical density	Circularity
Negative (I)	15.79	$38.62^b \pm 12.55$	$0.25^b \pm 0.02$	$0.77^b \pm 0.09$
Strong (II-a)	10.19	$25.89^a \pm 11.48$	$0.36^a \pm 0.02$	$0.59^a \pm 0.13$
Weak (II-b)	74.02	$44.12^c \pm 16.56$	$0.30^c \pm 0.02$	$0.69^c \pm 0.09$

[a,b,c]Values with different superscripts are significantly different at $P = 0.05$.

Figure 1 Cross-sections of *longissimus dorsi* muscle, periodic acid Schiff (PAS) staining, (times 200). Figure 1 presents the fibres with negative PAS reactive (I), fibres with strong PAS reactive (II-a) and fibres with weak and moderate PAS reactive (II-b). Type I fibres mainly comprise the middle-sized, round or ellipsoidal fibres distributed in clusters or dispersed among the PAS positive fibres (II-a, II-b). Type II fibres are composed a small group showing a strong PAS reaction and a large one showing moderately strong PAS reaction. The PAS-IIa fibres only occupy a small proportion with middle-sized and polygonal appearance, while the II-b fibres form the main portions for the muscle with difference appearance.
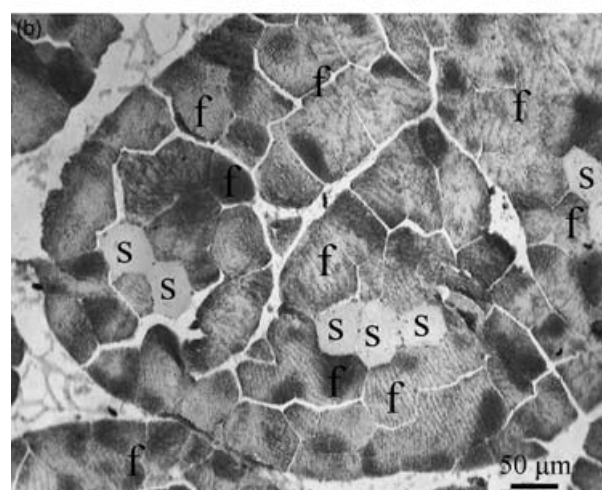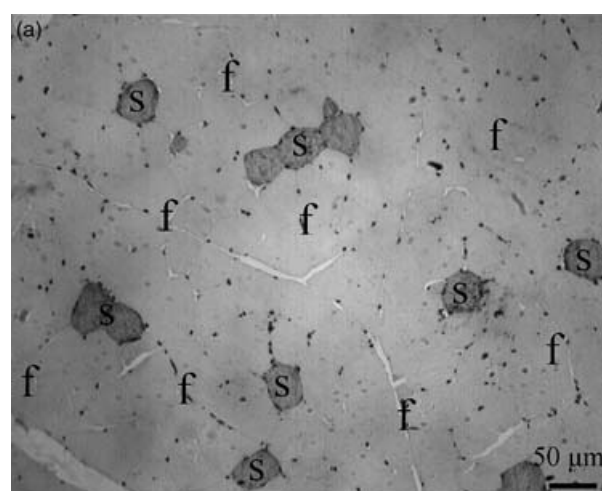

Figure 2 MHCs and MHCf immunohistochemistry in cross-sections of *longissimus dorsi* muscle. Plate (a) shows that the slow muscle fibres were stained with anti-MHCs monoclonal antibody. It was illustrated that the MHCs positive fibres (s) only make up a small proportion of the muscle. They were presented as small and round in appearance. The negative fibres (f) distributed around the positive fibres and separated them into clusters. Plate (b) shows that the fast muscle fibres (f) were stained with anti-MHCf monoclonal antibody. It was revealed that the fibres can be grouped as positive fibres (f) and negative fibre (s). The negative fibres distributed sparsely and separated by the positive fibres in clusters. $\times$200 magnification.

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*Msp*I, and the alleles were defined as follows: allele A = 275, 502 and 646 bp and allele B = 275, 370 and 502 bp (Figure 3). The PCR product incubated with *Hinf*I yielded five fragments (174, 200, 372, 503 and 646 bp): allele C = 174, 200, 372 and 646 bp bands and allele D = 174, 200, 372 and 503 bp (Figure 4). The *Rsa*I digested products contained 6 fragments (89, 162, 183, 240, 370

and 649 bp): allele E = 89, 162, 183, 240 and 649 bp and allele F = 89, 162, 183, 370 and 649 bp (Figure 5).

The results revealed that the genotypes of JP F_2 crossbred pigs can be clustered into three groups: AA/CC/EE, BB/DD/FF and AB/CD/EF. The frequency of AA/CC/EE, AB/CD/EF and BB/DD/FF was 0.1835, 0.5823 and 0.2342, respectively. As a result, the frequency of A/C/E and B/D/F is 0.4747 and 0.5254.
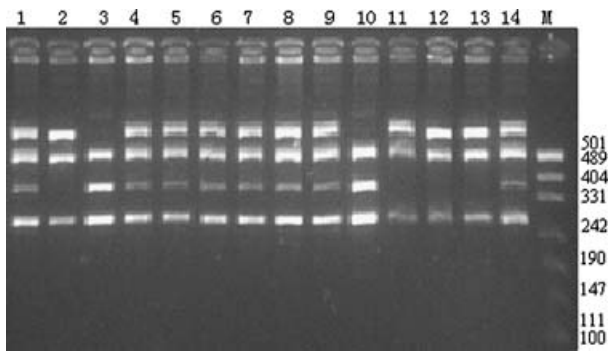
The effect of the CAST *gene on* post mortem *traits in JP* F_2 *pigs*

A GLM analysis was performed to interpret how the genotypes of the *CAST* gene influence *post mortem* traits of JP F_2 (Table 2). The genotypes show different association with the area ($P = 0.63$), WHC ($P = 0.15$), pH_{45m} value ($P < 0.01$) and conductivity ($P = 0.37$) of loin muscle.

The effect of genotypes of the CAST *gene on the muscle histological traits*

The effects of genotypes of the *CAST* gene on muscle histological traits are shown in Table 3. The data revealed that the genotypes of the *CAST* gene have different effects

Figure 3 Profile of *CAST* gene digested by *Msp*I. In the RFLP pattern, four fragments and two different cleavage patterns was resolved, The lane 1, 4, 5, 6, 7, 8, 9 and 14 were classified as AB genotype. All four bands (646, 502, 370, 275 bp) were presented on this lane; lanes 2, 11, 12 and 13 were AA genotype. There are only 646 bp, 502 bp and 275 bp three fragments in these lanes. The lanes 3 and 10 belonged to BB genotype, their profiles lacked 646 bp bands; M: Gene Ruler-pUC19 DNA/MspI Marker (Fermentas Inc., Burlington Ont., Canada).
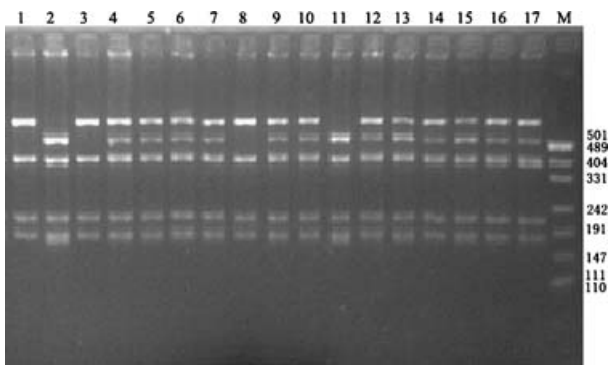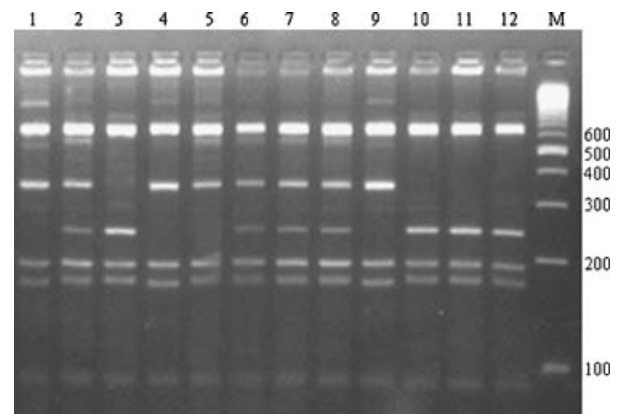

Figure 4 Profile of *CAST* gene digested by *Hinf*I. There are five fragments (174, 200, 372, 503, and 646 bp) and two different cleavage patterns detected using *Hinf*I. There are all of these five bands in lanes 4, 5, 6, 7, 9, 10, 12, 13, 14, 15, 16 and 17, thus it was classified as CD genotype. The 503 bp fragments did not exist in lanes 1, 3 and 8, thus, it was considered as CC genotype. The lanes 2 and 11 lacked the longest fragment and were sorted into DD genotype. M: Gene Ruler-pUC19 DNA/MspI Marker (Fermentas Inc., Burlington Ont., Canada). In addition, the samples were used in these patterns were different from Figures 3 and 5.

Figure 5 Profile of *CAST* gene digested by *Rsa*I. Six fragments (89, 162, 183, 240, 370, 649bp) could be distinguished from the RFLP patterns detected using *Rsa*I, two different cleavage patterns could be differentiated. The samples in lanes 2, 6, 7 and 8 were sorted into EF genotype, in which all bands were visible. The samples were loaded in lanes 3, 10, 11 and 12 were grouped into EE genotype and, in which the 370 bp fragment was invisible. The samples loaded in lanes 1, 4, 5 and 9 belong to FF genotype. In the patterns appeared no 240 bp bands. M: Gene Ruler-100bp+1.5kb DNA ladder (Biobasic Inc., Markham, Ont., Canada). In addition, the samples used in these patterns were different from Figures 3 and 4.

Table 2 *The effect of the* CAST *gene on* post mortem *trait of JP* F_2 *pigs (data are quoted as mean ± s.d.)*

Genotypes	No. observed	Area of loin muscle	Water-holding capacity	pH_{45m} value	Conductivity
AA/CC/EE	29	32.34 ± 3.36	0.60 ± 0.11	6.30[a] ± 0.21	2.99 ± 0.28
AB/CD/EF	92	31.49 ± 4.32	0.59 ± 0.06	6.19[b] ± 0.21	2.62 ± 0.11
BB/DD/FF	37	33.80 ± 3.92	0.57 ± 0.12	6.21[b] ± 0.14	2.81 ± 0.07

[a,b]Values with different superscripts are significantly different at $P = 0.05$.

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Table 3 *The effect of the* CAST *gene on histological traits of muscle of JP F$_2$ pigs (data are quoted as mean $\pm$ s.d.)[†]*

Genotypes	Diameter (μm)	Area (μm^2)	Circularity	Axratio	MHCs (%)	Density (per mm^2)	PCT (%)
AA/CC/EE	42.33[b] $\pm$ 0.06	1577.88[b] $\pm$ 229.35	0.66[b] $\pm$ 0.05	1.71[a] $\pm$ 0.05	5.63 $\pm$ 1.92	428.61[ab] $\pm$ 73.27	33.31[a] $\pm$ 12.67
AB/CD/EF	45.31[a] $\pm$ 0.46	1794.07[a] $\pm$ 336.42	0.69[a] $\pm$ 0.03	1.63[c] $\pm$ 0.07	6.13 $\pm$ 1.75	418.76[b] $\pm$ 91.48	27.53[b] $\pm$ 10.04
BB/DD/FF	45.29[a] $\pm$ 1.83	1791.45[a] $\pm$ 149.04	0.69[a] $\pm$ 0.02	1.66[b] $\pm$ 0.04	6.30 $\pm$ 2.85	444.57[a] $\pm$ 65.86	21.07[c] $\pm$ 10.79

[a,b,c]Values with different superscripts are significantly different at $P = 0.05$.
[†]Abbreviations are: MHCs is the number of MHC-positive muscle fibres. PCT is the percent of intramuscular connective tissue. Axratio is major axial-to-minor axial ratio.

on the diameter ($P < 0.01$), area ($P < 0.01$), circularity ($P < 0.01$) and axial ratio ($P < 0.01$) of muscle fibre. The analysed results also presented that the genotypes have significant effect on the percentage of connective tissue ($P < 0.01$), while no significant effect was found on the density ($P = 0.015$) and type ($P = 0.99$) of muscle fibres. The individuals with AA/CC/EE genotypes may have more ellipsoid muscle fibres, and the individuals with BB/DD/FF genotypes may generate more round-shaped muscle fibres. The proportion of intramuscular connective tissue in the muscle samples was higher in genotype AA/CC/EE and lower in genotype BB/DD/FF.

Discussion

Meat comprises skeletal muscles and connective tissue including any surrounding or interstitial adipose tissue. It is well documented that the muscle fibre is column shaped with a circular cross-section (Shen and Xu, 1984). The shape may primarily depend on the kind of muscle and its location. However, based on the measurement of more than 85 000 fibres in both paraffin-embedded and frozen sections from 270 pigs (unpublished), it was seen that the muscle fibres are mainly polygonal or ellipsoid in cross-section (Figures 1 and 2). In order to explain this difference, two parameters (circularity and axial ratio) are considered in this paper. We found only 0.4% muscle fibres with axial ratio less than 1.20 and circularity greater than 0.90. The average axial ratio is 1.63 ± 0.51 and average circularity is 0.72 ± 0.11, i.e. only a few fibres are round in cross-section. It follows from this that, in cross-section, the fibres are more polygonal or ellipsoidal than round; consequently, columns of muscle fibres are mainly prism-shaped or irregular rather than orderly.

The concentration of glycogen and oxidative ability are important factors in accessing meat quality (Karlsson *et al.*, 1999). PAS staining was employed to explore the intensity of PAS in muscles of JP crossbred pigs. The LM is close to the vertebral column, its main function being to maintain muscle tension rather than movement. The results detected by PAS staining indicated that the percentage of PAS-negative fibre is relatively low. This result may be caused by the distribution of muscle fibres and their main function. PAS-negative fibres are dispersed or present in clusters among and surrounded by PAS-positive fibres, which are

always towards the periphery in bundles of muscle. It remains to be proved whether this architectural pattern is related to muscle contraction.

By the MHC immunohistochemical method, the proportion of slow fibres (i.e. MHC-positive fibres) was found to be 5.8% in the LM in pigs (Bee *et al.*, 1999). We find that in JP F$_2$ pigs, myosin MHC-positive fibres account for 6% of the fibres in LM; the rest are MHCf-positive. These findings are consistent with those reported earlier (Lefaucheur *et al.*, 2002 and 2004). These slow fibres are also defined as primary fibres, and the number and size of these fibres determine muscle mass and individual size in mature animals (Wigmore and Stickland, 1983; Stickland and Handel, 1986; Dwyer *et al.*, 1993).

It is well known that it is difficult to distinguish between intramuscular connective tissue (IMCT) and intramuscular fat (IMF) by visual examination alone. As the proportion of IMCT increases, IMF also correspondingly increases. The relationship between IMF and tenderness in pork is controversial; some researchers reported a marked correlation whereas others found none (Avery *et al.*, 1996; Van Laack *et al.*, 2001). Pork with different IMCT levels may have originated from pigs with different genetic backgrounds. Our results also revealed that IMCT is related to the genotype.

The amplification products of the sequence between the sixth and seventh exon of *CAST* gene were digested by *Msp*I, *Hinf*I and *Rsa*I and discriminated two different cleavage patterns detected using each endonuclease. Cluster analysis of histological characteristics and cleavage patterns suggested that their PCR-RFLP profiles were similar. Only three genotypes could be distinguished. It must be pointed out that this result is possibly affected by sample size. It is also possible that this result is due to the conservatism of the *CAST* sequence in JP F$_2$ Pigs.

The results suggested that the restriction endonuclease polymorphism of *CAST* gene may play an important role in determining the composition and morphology of muscle fibres. Individuals with the AA/CC/EE genotype have a tendency to generate small, ellipsoid fibres, whereas those with BB/DD/FF or AB/CD/EF genotypes generate large and more rounded fibres with a lower proportion of IMCT.

Many factors such as fibre size, density and IMCT contribute to meat quality. Additionally, a unique characteristic of the skeletal muscle is its diversity, including the patterns in which individual muscle fibres are arranged and the

composition and heterogeneity of individual fibres. For these reasons, it is still not fully clear how the *CAST* gene influences meat quality. Our findings in this paper show that the *CAST* gene correlates with some histological characteristics, which may help in elucidating the *post mortem* effect of *CAST* gene in future research.

Acknowledgements

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