Institut für Tierwissenschaften, Abt. Tierzucht und Tierhaltung
der Rheinischen Friedrich – Wilhelms – Universität Bonn

Transcript abundance of myosin heavy chain isoforms and identification of candidate genes for body composition and meat quality in pigs

I n a u g u r a l – D i s s e r t a t i o n
zur Erlangung des Grades

Doktor der Agrarwissenschaft
(Dr. agr.)

der
Hohen Landwirtschaftlichen Fakultät
der
Rheinischen Friedrich – Wilhelms – Universität
zu Bonn

vorgelegt im Oktober 2006

von

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aus

Cantho, Vietnam

Zugl.: ITW; Bonn, Univ., Diss., 2006

D 98

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Tag der mündlichen Prüfung:	05. Dezember 2006

Dedicated to my parents, my brother and my wife

Quantifizierung der Transkription von Isoformen des Myosin Heavy Chain Gens und Identifikation von Kandidatengenen für Fleischleistungsmerkmale beim Schwein

In landwirtschaftlichen Nutztieren ist die strukturelle und funktionelle Diversität des Skelettmuskels durch eine Vielfalt von Myosin Isoformen gekennzeichnet. Die Tierproduktion kann durch ein besseres Verständnis der Muskelfasereigenschaften und der Gene, die diese beeinflussen, optimiert werden. In der vorliegenden Untersuchung wurden die Transkriptmengen von MyHC Isoformen quantifiziert und Kandidatengene für die Schlachtkörperzusammensetzung und Fleischqualität bei Schweinen identifizieren.

In den beiden kommerziellen Rassen Duroc und Pietrain wurden MyHC Typ II in mehr als der Hälfte aller MyHC Isoformen gefunden. Der Anteil an MyHC Typ IIb Transkripten war in den Kreuzungstieren DUPI (Duroc x Pietrain) und DUMI (Duroc x Berliner Miniaturschwein) ähnlich. Das in Vietnam beheimatete Schwein Mongcai zeigte im Vergleich dazu eine sehr geringe MyHC IIb Transkriptmenge sowie höhere Mengen an MyHC Isoformen. Beim Vergleich der Anteile der Fasern zwischen zwei extremen Muskelgruppen konnte ein signifikanter Unterschied des Anteils der Typ IIb Fasern bei unterschiedlichen Vollgeschwistergruppen der DUPI und der DUMI Population gefunden werden. Die Mikroarray Experimente ergaben drei Gene, die in der Tiergruppe mit kleinen Muskeln höher exprimiert sind. Für die Gene TNNC1, welches aufgrund einer vorangegangenen Mikroarray Hybridisierung ausgewählt wurde, und CKM, welches anhand von Literaturangaben ausgewählt wurde, konnten signifikant höhere Expressionen in Tieren der Rasse Pietrain verglichen mit Tieren der Rasse Mongcai gefunden werden. Ein SNP in der 3'UTR des FTH1 Gens war signifikant assoziiert mit der durchschnittlichen täglichen Zunahme (ADG) und der Scherkraft. Es wurde eine Assoziation des Haplotyps dieses Gens mit ADG, Fleischfarbe und pH-Wert detektiert. Ein SNP im Intron des TNNC1 Gens war mit der Kotelettfläche und anderen fleischbezogenen Merkmalen sowie der Leitfähigkeit assoziiert. Ebenso war ein SNP in der Promotorregion des CKM Gens signifikant assoziiert mit vielen Fettmerkmalen. Zusammenfassend konnte gezeigt werden, dass der Typ IIb für die Fasern ausschlaggebend ist, die zu einer Differenzierung in große und kleine Kotelettfläche beim Schwein führen. Die Gene FTH1, TNNC1 und CKM sind Kandidatengene für Wachstum, Kotelettfläche und Fettmerkmale.

Transcript abundance of myosin heavy chain isoforms and identification of candidate genes for body composition and meat quality in pigs

In farm animals, the structural and functional diversity of skeletal muscle is represented by the variety of myosin isoforms. Understanding muscle fiber characteristics and related genes will help to optimize the animal production. The present study has been carried out to quantify transcript abundance of MyHC isoforms and to further identify candidate genes for body composition and meat quality in pigs. Five pig breeds were included in this study. In both commercial breeds of Duroc and Pietrain, abundance of MyHC type IIb accounted for more than half of the MyHC transcripts. The proportion of MyHC type IIb transcripts was similar in the crossbred pigs DUPI (Duroc x Pietrain) and DUMI (Duroc x Berlin Miniature Pigs). The Vietnamese native pig, Mongcai, showed very low MyHC IIb transcript abundance compared to the other breeds and crosses. However, the Mongcai showed much higher relative abundance of the other MyHC isoforms. In comparison of fiber proportion between two extreme muscle groups, discordant sib-pairs in the DUPI and the DUMI population differed significantly in the percentage of type IIb fibers. In the hybridization of microarray experiment, four discordant sib-pairs of F2-DUPI animals were used. Three genes were shown to be highly expressed in the small muscle animal group and two of them, FTH1 and FHL1C were further validated by real-time RT-PCR. Moreover, the TNNC1 gene, derived from a preliminary microarray hybridization and the CKM gene, selected from literature were found to have significantly higher expression in Pietrain compared to Mongcai. For SNP detection and association analysis, two SNPs were detected in the 3'UTR of FTH1 gene. One of them had no effects on any performance traits, whereas the other SNP was statistically associated with average daily gain (ADG) and shear force. The constructed haplotype of this gene resulted in association with ADG, meat color and ultimate pH. For FHL1C, no SNP could be detected. In contrast, one SNP in the intron part of TNNC1 showed association with eye muscle area and other meat-related traits and conductivity. Finally, one SNP in the promoter region of CKM was significantly associated with many fatness traits. In conclusion, it is demonstrated that type IIb is the determinant fiber contributing to the differentiation of large and small eye muscle area in the pig. FTH1, TNNC1 and CKM genes are proposed as candidate genes for growth, loin eye muscle area and fatness traits, respectively.

Contents

List of abbreviations

A	: Adenine
A260	: Absorbance at 260 nm wavelength (UV light)
ADG 1	: Average daily gain, suckling (g/day)
ADG 2	: Average daily gain, from birth to test start (g/day)
ADG 3	: Average daily gain, from test start to slaughter (g/day)
ADG 4	: Average daily gain, overall from birth to slaughter (g/day)
A-FABP	: Adipocyte fatty acid-binding protein
ATP	: Adenosine triphosphate
bp	: Base pairs
BSA	: Bovine serum albumin
C	: Cytosine
$C1_{ML}$	: Conductivity, *Longissimus dorsi*, 45 min post mortem (mS/cm)
$C24_{ML}$	: Conductivity, *Longissimus dorsi*, 24 h post mortem (mS/cm)
$C24_{S}$	: Conductivity, *Semimembranosus* 24 h post mortem (mS/cm)
Cas	: Crk-associated substrate
CAST	: Calpastatin
cM	: Centimorgan
CSA	: Cross sectional area
CSTB	: Cystatin B
CTSB	: Cathepsin B
ddH_2O	: Distilled & deionized water
ddNTP	: Dideoxyribonucleoside triphosphate
dH_2O	: Deionized or distilled water
DMSO	: Dimethyl sulfoxide
DNA	: Deoxynucleic acid
dNTP	: deoxyribonucleoside triphosphate (usually one of dATP, dTTP, dCTP and dGTP)
DTT	: Dithiothreitol
E.coli	: *Escherichia coli*
EDTA	: Ethylenediaminetetraacetic acid (powder is a disodium salt)
EtBr	: Ethidium bromide

EtOH : Ethanol
FBAT : Family based association tests
FCR : Feed conversion ratio
FHL1 : Four-and-a-half LIM protein
Fig : Figure
FOS : c-fos proto-oncogene
FTH1 : Ferritin heavy chain
g : Gram
G : Guanine
GH1 : Growth hormone
h : Hour
HAL (RYR1) : Halothane (ryanodine receptor)
H-FABP : Heart fatty acid-binding protein
IGF2 : Insulin-like growth factor
Ile : Isoleucine
IMF : Intramuscular fat
IPTG : Isopropylthio-ß-D-galactoside
ISH : In situ hybridization
kb : Kilobases
KIT : c-kit proto-oncogene
l : Litre
LD : *Longissimus dorsi*
LEP : Leptin
Leu : Leucine
mA : Milliamperes
mATPase : Myosin adenosine triphosphatase
MC1R : Melanocortin receptor 1
MC4R : Melanocortin-4 receptor
MC_{Opto} : Meat color, Opto Star: meat color 24 hours post mortem (%)
mg : Milligrams
$MgCl_2$: Magnesium chloride
min : Minute
ml : Milliliters

mmole : Milimole
mRNA : Messenger RNA
MW : Molecular weight
MyHC : Myosin heavy chain
NaCl : Sodium chloride
ng : Nanograms
nm : Nanometers
OD260 : Optical density at 260 nm wavelength (UVlight); = A260
p.m. : Post mortem
PAGE : Polyacrylamide gel electrophoresis
PCR : Polymerase chain reaction
PGAM2 : Phosphoglycerate mutase 2
pH1 : pH value, 45 min post mortem
$pH1_{LD}$: pH value, *Longissimus dorsi*, 45 min post mortem
$pH24_{LD}$: pH value, *Longissimus dorsi*, 24 hours post mortem
$pH24_{S}$: pH value, *Semimembranosus* 24 hours post mortem
pH_u : pH ultimate
PIT1 : Pituitary transcription factor
PKM2 : Pyruvate kinase
pmol : Picomolar
QTL : Quantitative trait loci
RAMP1 : Receptor activity-modifying protein 1
RFLP : Restriction fragment length polymorphism
RN (PRKAG3) : Rendement Napole (Protein kinase AMP activated, γ3 subunit)
RNA : Ribonucleic acid
rpm : Revolutions per minute
SDS : Sodium dodecyl sulfate
Ser : Serine
SLIM1 : Skeletal muscle protein
SNP : Single nucleotide polymorphism
T : Thymine
TAE : Tris-acetate buffer
TBE : Tris- borate buffer

TE	: Tris- EDTA buffer
Thr	: Threonine
TNF	: Total number of fiber
tRNA	: Transfer RNA
U	: Units
UTR	: Untranslated region
UV	: Ultra-violet light
V	: Volts
v/v	: Volume per volume
W	: Watts
w/v	: Weight per volume
X-gal	: 5-Bromo-4-chloro-3-indolyl-beta-D-galactoside

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1 Introduction

Growth in general, the deposition of muscle and fat tissue, is a result from a multitude of biological processes affected by many factors. Muscle growth is one of the most important goals in animal production and meat science since it contributes to the economically important outputs of animal agriculture. In quantitative terms, growth is the increase in living substance or protoplasm including cell multiplication, cell enlargement and incorporation of material from the environment (Gu et al. 1992). Muscle fibers are the major constituent of a given muscle and thus muscle mass is largely determined by the number and the size of those fibers (Rehfeldt et al. 2004). Skeletal muscle growth can be divided into two stages: prenatal myogenesis and postnatal development. During embryonic period, myogenic progenitor cells proliferate to myoblasts, which differentiate and fuse to form multinucleated myotubes and later modulate to become muscle fibers (Rehfeldt et al. 2000). These basic events are involved in muscle-specific gene expression (Muscat et al. 1995). In postnatal growth, the increase of muscle mass is mainly determined by the increase of muscle fiber size, a process accompanied by the prolificative activity of satellite cells, which is known as a source of new nuclei incorporated into the muscle fibers (Rehfeldt et al. 2004). Muscle fiber is therefore considered an important factor influencing many of the peri- and post-mortal biochemical processes and thereby meat quality (Klont et al. 1998).

In meat producing animals, growth rate and the kind of tissue deposited are major effects on economic important traits reflecting the action and interactions of many different physiological pathways. Several economic traits have been explored in pig industry including fattening traits (daily gain, feed efficiency), body composition traits (meat content, fat content, loin eye area) and meat quality traits (color, water holding capacity, pH, tenderness, intramuscular fat). These traits are caused by a number of genotypic and environmental factors. From the view of molecular genetics, genotypic effects rely on the variation of the nucleotide sequence in the genome that either influences the expression level of a given gene or the functional properties of the encoded proteins. Contributing greatly to this variation are the effects of extreme breeds and the structural and functional properties of muscle itself.

The finding of genes responsible for genetic variation in the traits of interest in animal species is of importance in genomic analysis (Rothschild and Soller 1997). Currently, there are two different approaches known as QTL mapping and candidate gene analysis. While the first technique is to discover genomic regions related to quantitative traits, the other one focuses on detection of mutations in candidate genes and their possible association with economical production traits (Óvilo et al. 2002) and thereby exploits information from previous cellular, biochemical or physiological functional studies to target a gene of interest. Candidate genes can be derived based on knowledge of the function of the gene product (direct biological candidate) or its specific expression pattern (functional candidate).

The present study was undertaken with the following objectives:

1. To quantify transcript abundance of myosin heavy chain isoforms in commercial and local pig breeds and further figure out the expression profile of these isoforms in low and high performing pigs in two F2-resource populations.
2. To identify differentially expressed genes in pigs with extreme muscles and in breeds with extreme muscularity as candidate genes for histochemical and biochemical muscle properties.
3. To detect single nucleotide polymorphisms and analyze association of candidate genes with body composition and meat quality traits in pigs.

2 Literature review

In farm animals, the structural and functional diversity of skeletal muscle is represented by a variety of myosin isoforms. Understanding muscle fiber characteristics will help to optimize the efficiency of the muscle growth and meat quality, the two important concerns in animal production. To this point, fiber classification, myosin heavy chain (MyHC) expression, their role in muscle development and meat quality traits and possible factors influencing the fiber proportions are briefly described in the next section.

2.1 Classification and existence of muscle fibers in mammalian species

The mammalian skeletal muscle is composed of a heterogeneous collection of fiber types with polynucleated and elongated cells that can be classified according to their MyHC isoforms, energy metabolism and contractile properties (Lefaucheur et al. 1998, Picard et al. 2002). Depending on different techniques in classification, muscle fibers can be either grouped together by this technique or placed in different categories by another method. Table 1 displays the muscle fiber typing with different classification schemes.

Table 1: Classification of skeletal muscle fiber types, modified from Spangenburg and Booth (2003)

Classification scheme	Muscle fiber types			
Myosin heavy chain	Type I	Type IIa	Type IIx	Type IIb
Contractile speed	Slow	Fast	Fast	Fast
Metabolic	Oxidative	Oxidative	Glycolytic	Glycolytic
Color	Red	Red	White	White
Fiber CSA	Small	Medium	Large	Large
Mitochondria	Many	Many	Few	Few
Capillaries	Many	Many	Few	Few
Fatigue resistance	High	Intermediate	Low	Low
Function	Postural, endurant movement	Postural, endurant and fast movement		Fast movement

Myosin is a fundamental structural and functional component of all skeletal muscles, and about 1/3 of the total muscle proteins are from myosin making it the vast majority of the contractile apparatus of muscle fibers (Picard et al. 2002). Four out of eight isoforms known in mammals have been identified in porcine muscle according to their specific expression of MyHC (Chang and Fernandes 1997). Based on their metabolic and myosin adenosine triphosphatase (mATPase) activity, these fibers, each encoded by an individual gene, are categorized as slow-oxidative and fast-glycolytic or slow/I and IIb fibers, standing for extreme metabolic profiles. The IIa and IIx fibers are defined as intermediates with the transition that IIa fibers are more similar to slow/I type and IIx fibers are more in relation to IIb fibers (Chang et al. 2003, Pette and Staron 2000). In addition, skeletal muscle can also be classified as light or dark according to its fiber composition, i.e. fast-glycolytic fibers are prominent in light muscle while slow-oxidative fibers dominate in dark muscle. Moreover, owing to their metabolic characteristics, muscle fibers can be distinguished as red (oxidative) and white (glycolytic) but this distinction is applied only with type I and IIa because type IIb shows heterogeneous features, in which 15% of IIb fibers were reported red (Larzul et al. 1997). In the pig, gene coded for type I fiber is found on chromosome 7 in one cluster, whereas genes responsible for embryonic, IIa, IIx, IIb, neonatal and extraocular fibers are located on chromosome 12 (Davoli et al. 2002). Different locations give them distinct function. Slow-twitch fibers (type I), which contain high level of slow contractile proteins, high volume density of mitochondria, high levels of myoglobin, high capillary densities and high oxidative enzyme capacity (Spangenburg and Booth 2003), are responsible for posture maintenance. On the other hand, fast glycolytic fibers (type IIb) with low volume of mitochondria, high glycolytic enzyme and myosin ATPase activity, high level of fast contractile protein and increased rate of contraction (Spangenburg and Booth 2003) are more involved in producing movement.

Although there are eight isoforms of MyHC existing in two clusters in mammalian skeletal muscle (Shrager et al. 2000, Weiss et al. 1999a), not all are expressed in different species. By using conventional methods (electrophoresis, histochemical mATPase and anti-myosin monoclonal antibodies), the presence of four isoforms I, IIa, IIx and IIb were first detected in adult skeletal muscle of rats (Bar and Pette 1988, Schiaffino et al. 1989), mouse and guinea pigs (Gorza 1990) and rabbits (Aigner et al. 1993). In some larger animals, evidences of three adult fast isoforms were reported in

pigs (Lefaucheur et al. 1998) and in llama (Graziotti et al. 2001). On the contrary, isoform IIb was described to be absent in skeletal muscle of large mammals such as goat (Arguello et al. 2001), horse (Chikuni et al. 2004a, Eizema et al. 2003) and dog (Smerdu et al. 2005). In ovine muscle, the proportion of type I and type II fibers all together was identified (Sazili et al. 2005), but the existence of IIb fibers has not been well documented. In cattle, most researchers found no evidence of type IIb expression (Chikuni et al. 2004b, Muroya et al. 2002, Tanabe et al. 1998) except Picard et al. (1998), who could separate three bands of the fast isoforms in bovine muscle. It is noteworthy that non-expression of IIb does not mean an absolute absence but probably this isoform is present in a certain muscle. Examples can be found in additional studies, which showed that the expression of type IIb is specialized in eye muscle but not in trunk and limb, and that this type is evidently expressed only in extraocular muscles (Maccatrozzo et al. 2004, Toniolo et al. 2005). Finally, the classification of human skeletal muscle fibers is rather confusing. Earlier studies by Smerdu et al. (1994) and Ennion et al. (1995) documented the absence of IIb isoform, but later by DNA analysis, Weiss et al. (1999b) provided incompatible outcomes. Indeed, what has been named IIb in human was actually type IIx/d, an intermediate contractile speed between IIa and IIb isoforms (Hilber et al. 1999) and thereby it is likely that human muscles do not express the fastest MyHC IIb (Pette et al. 1999).

2.2 Prenatal and postnatal muscle development and expression of muscle fiber types

Muscle fibers originate from myogenic precursor cells or myoblasts followed by proliferation to form myotubes and finally differentiation into muscle fibers. The pattern of muscle fiber type formation, development and ontogenesis has been published (Lefaucheur et al. 1995, Picard et al. 2002, Schiaffino and Reggiani 1994). An insight into temporal regulation of MyHC isoforms in prenatal porcine skeletal muscles was reviewed by da Costa et al. (2003) and Lu et al. (1999). Clearly, in prenatal mammalian muscles, expression of embryonic, prenatal and slow/ß isoforms are dominant (da Costa et al. 2003). At birth, the expression levels of embryonic and prenatal genes are very low and replaced by other isoforms known as IIa, IIx and IIb fibers and thus making them the three fiber types together with type I to be expressed in skeletal muscle in

postnatal growing pigs (Lefaucheur et al. 2002). Representations of fiber type differentiation from embryonic to adult animals and postnatal development of fiber diameter and total fiber number are illustrated in Figure 1 and Figure 2, respectively.

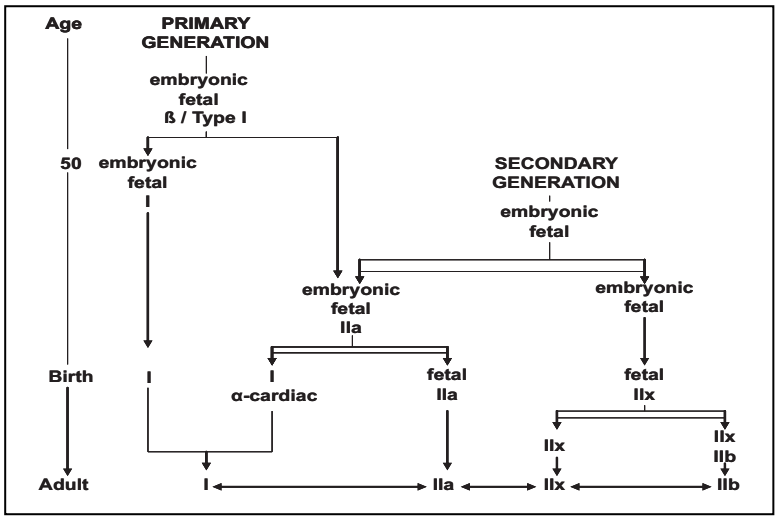


Figure 1: Schematic representation of fiber differentiation in developing skeletal muscle based on myosin heavy chain isoform transitions, adapted from Lefaucheur and Gerrard (1998)

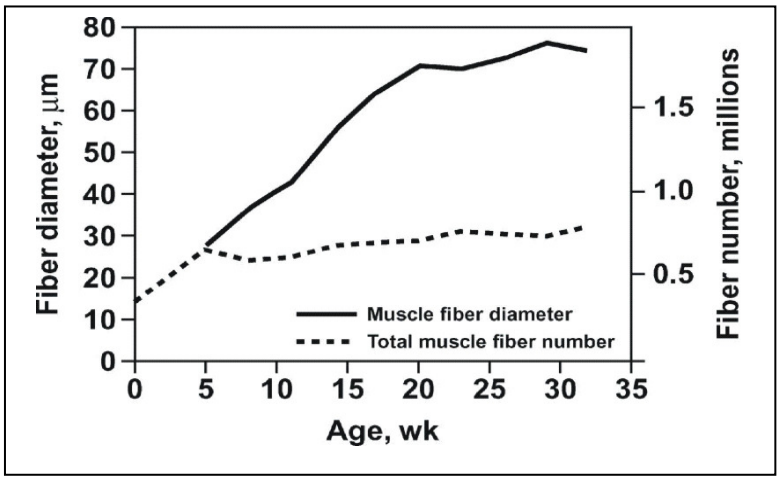


Figure 2: Postnatal development of fiber diameter and total fiber number per cross section in the semitendinosus muscle of German Landrace pigs, adapted from Rehfeldt and Kuhn (2006)

2.3 Factors affecting porcine muscle fiber types

There are several factors that can contribute to alter the muscle fiber composition in farm animals (Table 2). Some of them genetically originate from species, breed, sex, muscle and individuals, whereas others are environmental factors such as nutrition, temperature, exercise and some growth promoters.

Table 2: Intrinsic and extrinsic factors affecting histological traits, adapted from Lefaucheur and Gerrard (1998)

Item	Total number of fibers	Fiber type	
		Percentage	Diameter
Muscle	***[1]	***	**
Species	***	**	*
Breed	***	**	*
Sex	NS	**	**
Fetal nutrition	**	*	**
Postnatal nutrition	NS	*	**
Ambient temperature	NS	**	*
Individual	**	**	**
Exercise	NS	**	**
Growth promoters			
Growth hormone	NS	NS	**
β-Agonists	NS	**	**
Steroids	NS	**	**

[1] Intensity of effects (***, very strong; **, strong; *, medium; NS, not significant)

2.3.1 Muscle

The composition of fiber types varies between different muscles (Figure 3). Their location and function directly modify the proportion of fiber type in farm animals. The distribution of pig muscles is unique and highly organized, in which deep muscles contain more type I fibers surrounded by an internal rosette of IIa fibers and an external ring of IIb fibers (Lefaucheur and Vigneron 1986). Functionally, type I fibers are more involved in maintaining posture while IIb fibers are responsible for rapid movement

(Ono et al. 1995). In commercial breeds, the proportion of IIb/IIx fibers of *Longissimus dorsi* (LD) and *Psoas* are highest compared with the other fibers as pointed out by many authors (Chang et al. 2003, Gondret et al. 2005, Karlsson et al. 1993, Lefaucheur et al. 2004, Müller et al. 2002). The same tendency was also noticed in *Biceps femoris, Quadriceps femoris* (Karlsson et al. 1993) and *Adductor* (Ruusunen and Puolanne 1997). On the other hand, examples of high degree of type I and IIa fibers can be seen in Lefaucheur et al. (2004) and Gondret et al. (2005) for the *Rhomboideus* muscle.

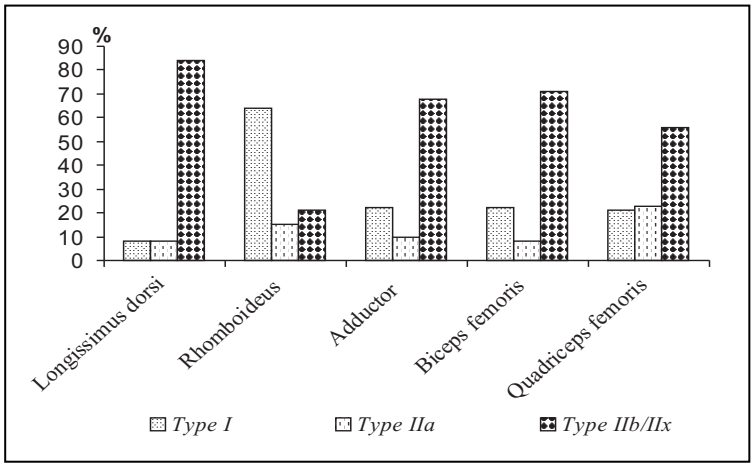


Figure 3: Percentage of muscle fibers in different muscles in the pig (Gondret et al. 2005, Karlsson et al. 1993, Ruusunen and Puolanne 1997)

2.3.2 Species

Muscle mass fluctuations in different species are related to the total number of fibers, the proportion and distribution of a given fiber. In farm animals, variations in body size are more related to total number of fibers (TNF) than fiber cross-sectional area (CSA), but these variations are not sufficient to reflect the variation of muscle fiber size (Rehfeldt et al. 1999) and thus species-specific differences in muscle mass are primarily due to differences in the TNF. Moreover, advances in technologies particularly in biochemistry, microscopy and molecular biology have allowed scientists to discover the diversity of muscle fiber in mammalian species. Various levels of MyHC expression have been identified in human, pig, dog, cattle, sheep, goat, rabbit, horse, llama and mouse. Details of fiber type composition present in some species are shown in Table 3.

Table 3: Muscle fiber type composition in different species

Species	Muscle fiber types (%)				Reference
	Type I	Type IIa	Type IIx	Type IIb	
Cattle *(gluteus medius)*	39.0	37.0	----------24.0----------		Agüera et al. (2001)
Sheep *(Longissimus dorsi)*	8.9	----------91.1----------			Sazili et al. (2005)
Goat *(M. semitendinosus)*	42.0	----------58.0----------		0.0	Arguello et al. (2001)
Rabbit *(Limb)*	4.1	32.6	---------63.3--------		Yamazaki et al. (2003)
Mouse	0.0	30.0	0.0	70.0	Burkholder et al. (1994)

2.3.3 Breed

The composition of muscle fiber types in different pig breeds is listed in Table 4. In general, genetic differences among different breeds lead to the variation in mature body size and the age, at which, pigs can be slaughtered. In comparison between the number and types of muscle fibers in large and small pig breeds, Stickland and Handel (1986) announced that the responsible causes for muscle size variation between Large White (LW) and miniature pigs are due to the difference in myofiber number, a factor fixed before postnatal growth (Stickland and Goldspin 1973). In addition, it appeared that pigs exhibiting postnatal increases in myofiber size are more related to age than to live weight. The mechanism to which less muscle development in genetically small animals is different from that exhibited by nutritional deprivation animals *in utero* (Stickland and Handel 1986), and consequences of this difference are therefore reflected in chemical properties of the constituents of muscle fibers. In comparison between miniature and LW pigs, the same authors recorded a higher content of type I fibers in the latter breed, which may support their increased weight and accord with the hypothesis that at similar stage of growth, there are great differences across breeds and fiber sizes. Finally, muscle of wild pigs was reported to contain higher area percentage of IIa and conversely lower percentage area of IIb fibers than those from the same muscle of domestic pigs (Ruusunen and Puolanne 2004).

Table 4: Muscle fibers: breed-specific distribution and detection methods

Pig breeds	Muscle fiber type (%)				Method[1]	References
	I	IIa	IIx	IIb		
Modern						
Large White	6.2	7.5	23.4	62.9	Realtime PCR	Lefaucheur et al. (2004)
Large White	11.4	4.0	34.7	52.4	ISH	Chang et al. (2003)
Duroc	14.7	3.2	29.6	51.9	ISH	
Landrace	13.2	9.0	------77.9------		mATPase	Ruusunen and Puolanne (1997)
Yorkshire	9.9	8.6	------81.5------		mATPase	
Pietrain	8.5	6.1	------85.2------		mATPase	Müller et al. (2002)
Traditional						
Berkshire	10.2	3.6	30	55.7	ISH	Chang et al. (2003)
Tamworth	12.9	6.1	31.5	49.7	ISH	
Meishan	8.3	13.4	61.1	17.1	Realtime PCR	Lefaucheur et al. (2004)
Hampshire	15.2	9.4	------75.3------		mATPase	Ruusunen and Puolanne (1997)
Wild boar	7.0	8.8	------84.2------		mATPase	Müller et al. (2002)
Crossbred						
Pi x LW	13	10	------75.0------		mATPase	Fiedler et al. (1999)
Du x (Y x LW)	7.9	11.9	------80.2------		mATPase	Ryu et al. (2004)
DUMI	15.3	12.2	------71.3------		mATPase	Fiedler et al. (2003)

[1] Four fiber types can be detected by either Realtime PCR or ISH while only three isoforms are distinguished by mATPase

2.3.4 Sex

There have been contradictory documentations on the distinction between females and males in term of proportion of muscle fibers. Generally, females have larger fibers than castrated males and as a result, sex has been mentioned to affect on the cross-sectional area but not on the proportion of each fiber (Bee 2004, Larzul et al. 1997, Solomon et al. 1990, Weiler et al. 1995). In addition, Lefaucheur and Gerrard (1998) demonstrated a significant decrease of relative fiber area of type I fiber in females compared to intact LW males, which may indicate that castration decreases relative area of type I fibers. Nevertheless, Rehfeldt et al. (2000) evidenced smaller values of IIa and IIb fibers in boars in comparison to female pigs and this implies a higher numbers of muscle fiber in male pigs because the weight of the LD muscle was similar.

2.3.5 Birth weight

The effect of birth weight on the muscle fiber performance has been mentioned by many researchers. Indeed, a tendency revealed that lower total fiber number in piglets is associated with low birth weight (Dwyer and Stickland 1991, Gondret et al. 2005, Wigmore and Stickland 1983). Most of the variations were due to a difference in the number of secondary myofibers that formed around each primary myofibers. However, results from other studies did not observe any association between birth weight and TNF and thereby rejected this suggestion (Dwyer et al. 1993, Handel and Stickland 1987). Although birth weight was indicated to have an association with enlarged muscle fiber area (Gondret et al. 2006), its influence on muscle fiber composition of LD and *Rhomboideus* muscle was not established (Bee 2004, Gondret et al. 2005). Similarly, Rehfeldt and Kuhn (2006) stated that the ranking of fiber number at slaughter was almost the same as at birth, with low fiber numbers in low birth weight and high numbers in high birth weight piglets. Because no differences were observed in the frequencies of different fiber types, Rehfeldt and Kuhn (2006) concluded that postnatal fiber differentiation is independent of birth weight.

2.3.6 Prenatal nutrition

In general, the nutritional manipulation has little effect on the early period of myogensis, a stage involved in the differentiation of primary muscle fibers. However, Rehfeldt and Kuhn (2006) emphasized that nutrition may change the number of primary fiber differentiation possibly because of indirect influence on the placenta development (Dwyer et al. 1995). Moreover, a decreased maternal nutrition before fiber formation in ewes was observed to alter the muscle characteristics in newborn lambs in a way that these lambs had fewer fast fibers and thereby significantly higher slow fibers (Fahey et al. 2005). In pigs, between 25 to 90 days of gestation, the differentiation and hyperplasia of secondary fibers have been demonstrated as a cause by undernutrion, which can lead to runting, a decrease in muscle fiber numbers, especially secondary fibers (Foxcroft et al. 2006, Handel and Stickland 1987, Wigmore and Stickland 1983). Likewise, findings on the relationship between overnutrition and muscle fiber

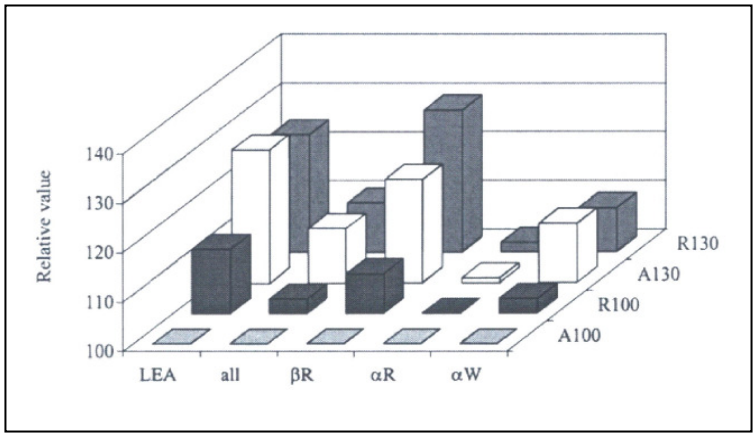
characteristics are still unclear. While Dwyer et al. (1994) reported that overnutrition of the sow between 25 to 50 days of gestation might increase the TNF in developing pigs; Nissen et al. (2003) did not find any benefits on muscle fiber number and area in the offspring when increasing maternal nutrition of sows from day 25 to 50 or day 25 to 70 of gestation.

2.3.7 Postnatal nutrition

There has been much attention on the role of postnatal nutrition on muscle development. Undernutrion was demonstrated to account for a reduction of cross-sectional area of future fast-twitch glycolytic fibers in LD muscle (Lefaucheur et al. 2003). Chilibeck et al. (2005) carried out an experiment on rapidly growing gilts, in which limited overfeeding of 75% more energy than needed for weight maintenance was offered at two stages, from day 1-7 (early luteal phase) and from day 8-15 (late luteal phase) of the estrous cycle. Results showed that muscle fiber area and fiber type composition were independent on restricted overfeeding at any time exclusive of a significant decrease in type IIa fiber percentage over time. Other experimental results were presented by Čandek-Potokar et al. (1995), who failed to evaluate any effect of restricted feeding (app. 30%, started from 30 till 100 kg) on fiber number, cross-sectional area, diameter and relative area of fast-twitch oxidative and fast-twitch glycolytic fibers. However, a significant difference was noted in case of slow-twitch oxidative fibers, in which restricted pigs had larger cross-sectional area, diameter and relative area than those from *ad libitum* fed pigs. Supportably, White et al. (2000) unraveled an association between postnatal nutritional status and type I fiber expression both at mRNA and protein levels. Also, restricted feeding (50% of *ad libitum*) at an early stage (between 3 and 7 weeks of age) had no influence on myofiber type composition in LD, but did lead to a remarkable increase in type I fiber proportion in the red rhomboideus muscle (Harrison et al. 1996). An assumption for these findings is, because the energy usage per unit tension is lower in type I fibers, as a result, a selective increase in type I proportion in muscle during the period of reduced available energy would be physiologically relevant to spare energy (Harrison et al. 1996, Lefaucheur and Gerrard 1998).

2.3.8 Age / slaughter weight

At birth, muscle fibers are oxidative and the relative number of slow-twitch fibers continues to increase until 8 weeks after (Lefaucheur and Vigneron 1986). The proportions of white fibers were observed to intensively increase up to 4 months of age together with the rapidly increased size and continue at a slower rate afterward (Kłosowska et al. 1985). Surprisingly, although IIa fibers are minor in porcine skeletal muscle, their relative fiber type-restricted expression was highest among 4 isoforms at both stages six weeks and 22 weeks postnatal (da Costa et al. 2002). A relation of age and muscle fibers was additionally recorded by (Čandek-Potokar et al. 1999, Correa et al. 2006) in such a way that increasing weight and age at slaughter did change the cross-sectional area but not the numerical percentage of any fibers. Figure 4 depicts the effect of slaughter weight on CSA of muscle fibers.



A: ad libitum

R: restricted 30%

LEA: loin eye area

βR: slow-twitch oxidative

αR: fast-twitch oxidative

αW: fast-twitch glycolytic

Figure 4: Relative values (value 100: group A100) for loin eye muscle area (LEA), cross-sectional area of all fibers, βR, αR and αW fibers according to experimental groups of pigs from 100 to 130 kg slaughter weight, adapted from Čandek-Potokar et al. (1999)

2.3.9 Other factors

Other factors that may have an impact on muscle fiber characteristics include individual, physical activity and ambient temperature and growth-promoting agents. Animals of the same breed and reared in the same environment also show large

variation in fiber type composition. An example for this is the variation within litter of Danish Landrace x Large White pigs, as described by Nissen et al. (2004). In that experiment, they grouped and slaughtered the animals by litter, at the same age, according to the body weight: heaviest, middle and lightest weight. It was found that, intralitter growth performance varied largely as a result of differences in both the number and growth rate of muscle fibers following the trend that heavy pigs had higher TNF than middle and lightest weight pigs, which did not differ from each other. Climate conditions and physical exercise can also play an important role in pig performance when the pigs are raised in an outdoor production system. Animals born outdoor at low temperature had a higher percentage of type I but lower percentage of IIa fibers in the *Longissimus* muscle. Pigs finished in outdoor environment were indicated to have higher proportion of IIa fibers in both *Longissimus* and *Semimembranosus* muscle and lower proportion of IIb/IIx fibers (Gentry et al. 2004). An increase of type I fiber percentage in pigs long-term exposed to cold temperature is generally accompanied by an increase in oxidative metabolism (Lefaucheur et al. 1991). Moreover, outdoor pigs have more spontaneous activities leading to a shift of muscle fibers from IIb to IIx to IIa to I, which can explain the more IIa and less IIb/IIx in muscle compared with indoor pigs (Lefaucheur and Gerrard 1998). In addition to some environmental factors, growth promoters such as growth hormone, β-agonists and steroids can influence muscle fiber composition of farm animals. An excellent review regarding to these promoting agents is available (Rehfeldt et al. 1999).

2.4 Significance of muscle fiber types for growth performance

There have been attempts to investigate the relationships between muscle fiber frequencies and muscle size or muscle performance in pigs (Henckel et al. 1997, Swatland 1982). It is well documented that TNF, CSA and muscle length are important parameters for muscle characteristics as well as muscle weight (Lefaucheur and Gerrard 1998).

2.4.1 Total number of fibers and muscle growth

In most cases, the TNF, a factor positively related to muscle growth potential in pigs, remains unchanged after birth (Wigmore and Stickland 1983) and thus within a muscle, muscle fiber hypertrophy is dependent on the TNF. Kłosowska and Fiedler (2003) suggested evidence that higher level of hypoplasia of individual fibers or higher number of TNF can be responsible for higher meat content. This was in line with previously reported data evidencing a positive correlation between TNF and carcass lean meat content (Dwyer et al. 1993, Handel and Stickland 1988). Fiedler et al. (2004) investigated that live weight and loin muscle area are positively related to TNF and frequency of white fibers. In Meishan pigs, a lower TNF was shown to result in smaller S*emitendinosus* muscle at birth and later to affect the proportion of white fibers (Lefaucheur and Ecolan 2005). All together, it is clear that TNF is significantly important to muscle size or muscle performance.

2.4.2 Fiber cross-sectional area and muscle performance

On the other hand, the correlation between CSA and muscle mass is controversial, probably because of the fact that lean meat content is mainly influenced by TNF, a highly variable trait (Lefaucheur 2006). For example, Henckel et al. (1997) reported a positive correlation between muscle gain and the oxidative enzyme citrate synthesis and the muscle capillarity of Large White and Landrace pigs. Additionally, daily gain was found to be tightly linked with CSA of type I fibers (Ruusunen and Puolanne 2004). Conversely, Larzul et al. (1997) were not able to point out any significant connection between CSA of individual fibers and average daily gain within the Large White breed. In most studies, glycolytic fibers are shown to exhibit the large CSA implying that, for a given TNF, an increase in muscle weight would be expected when the proportion of glycolytic fibers increases (Lefaucheur and Gerrard 1998). Moreover, the relationship between fiber diameters and perimeters was highly and positively related to muscle fibers and hence, it was concluded that fiber type proportion is more closely involved in their numerical abundance than their CSA (Ryu et al. 2004). Linear phenotypic correlation coefficients among those elements are presented in Figure 5. In this figure, it

is clearly shown that the muscle cross-sectional area is positively correlated with both the size and the number of muscle fibers. However, these values are in a wide range, which means there is a large variation in the number of total fibers as well as their growth rates even within the same litter (Nissen et al. 2004). Furthermore, the negative correlation between fiber number and fiber size can be explained by the equal distribution of energy in all fibers (Rehfeldt et al. 2000).

2.4.3 Myofiber length and muscle mass

Studies of myofiber length in different fibers have not been well documented, except for a recent research done by Christensen et al. (2006), who found shorter sarcomere in type IIb than in type I fibers isolated from LD muscle. This might contribute to the variation or differences in the mechanical properties of muscle fibers.

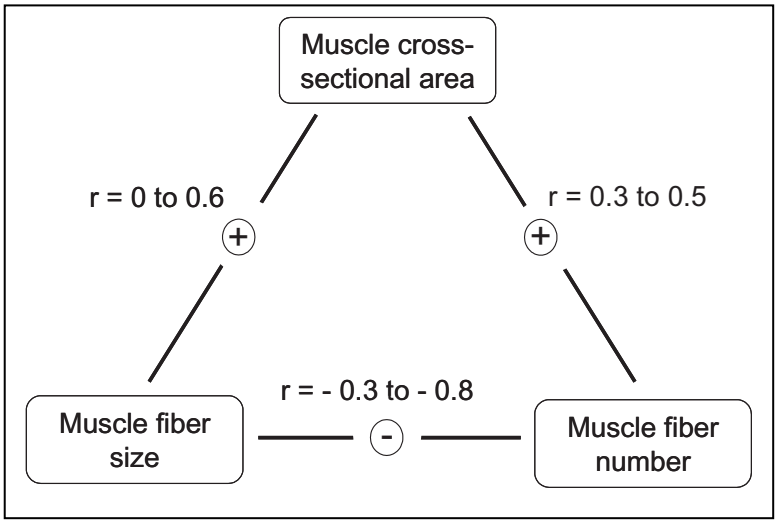


Figure 5: Relationships by linear phenotypic correlation coefficients between muscle cross-section area, muscle fiber size and muscle fiber number per cross-section, adapted from Rehfeldt et al. (2000)

2.5 Significance of muscle fibers on meat quality

Muscle fiber composition is in one way affected by growth rate and, in the other, itself affects the muscle mass. Meat quality can be defined as a combination of fresh meat or the degree of satisfaction of consumers to a given meat. Meat quality is assessed by

measuring biophysical and chemical properties, such as water holding capacity, color and light reflectance, pH, pigment content, shear force, intramuscular fat content and protein extractability (Karlsson et al. 1999) as well as eating quality and post mortem (p.m.) maturation of meat (Lefaucheur and Gerrard 1998). The correlation coefficients between muscle fibers and these factors appear in the wide ranges, some of which are listed in Table 5.

Table 5: Correlation coefficients (r) between proportion of muscle fibers and meat quality traits [(1)]

Item	Muscle fiber types			
	Type I	Type IIa	Type IIx	Type IIb
pH24	-0.46 to 0.20	0.02 to 0.28	0.10 to 0.30	-0.23 to 0.11
Drip	-0.04 to -0.06	-0.28 to 0.00	-0.40	-0.04 to 0.36
Shear force	-0.34 to 0.23	-0.05 to 0.13	not available	-0.04 to -0.13
IMF	0.00 to 0.04	-0.04 to -0.31	-0.03	0.03 to 0.21
Lightness (*L**)	-0.12 to 0.02	-0.07 to 0.14	-0.09 to 0.10	-0.19 to 0.27
Redness (*a**)	-0.08 to 0.44	-0.10 to 0.62	0.03	-0.14 to -0.48
Yellowness *(b*)*	-0.16 to 0.29	0.04 to 0.33	0.36	-0.35 to 0.07
Juiciness	-0.01 to 0.09	-0.20 to 0.09	not available	0.05
Tenderness	-0.06 to 0.10	-0.22 to 0.05	not available	0.01 to 0.06

[(1)] (Chang et al. 2003, Gentry et al. 2004, Henckel et al. 1997, Larzul et al. 1997, Ryu and Kim 2005)

2.5.1 Water holding capacity

A possible definition of water holding capacity (WHC) is the ability of meat or meat systems to retain all water or part of its own and/or added water (Honikel 2004). This ability relies on the handling method and the state of the system, and is important because muscle contains approximately 75% water and other components such as protein (20%), lipids (5%), carbohydrate (1%) and vitamins and minerals (1%) (Huff-Lonergan and Lonergan 2005). In highly processed pork products, the higher the WHC is, the more valuable the pork will be. However, the level of WHC varies among muscles likely because of the differences in postmortem degradation of intermediate filament proteins. Therefore, it was hypothesized that greater WHC would be achieved when rapid degradation of intermyofibril linkages (desmin) occurred (Kristensen and

Purslow 2001). The understanding about the relationship between muscle fiber type distribution and WHC is still poor, although WHC is one of the major factors directly related to fresh pork, with pale, soft and exudative (PSE) and dark, firm and dry (DFD) being the extreme types of meat. According to metabolic rate, Ryu and Kim 2006 (2006) recently determined a difference in type I fiber composition with an increased percentage from fast to slow metabolic group. A lower percentage of IIa fibers in PSE than in DFD pork was also mentioned. Particularly, fast-glycolyzing PSE pork contained the highest proportion of IIx/IIb fiber, which may be more prone to undesirable pork because of its anaerobic nature, greater glycogen content and lower ultimate pH (Ryu and Kim 2006, Solomon et al. 1998). However, as suggested by Ryu and Kim (2006), further research is needed to clarify the functions of pure IIx and IIb fibers on meat quality traits.

2.5.2 pH

Measurements of pH1 (45 min p.m.) and ultimate pH (pH_u, 24h p.m.) can indicate the rate and extent of p.m. glycolysis and are good indicators of meat quality. After slaughter, glycogen is converted into lactic acid, which is accumulated in the muscle. As a result of increasing lactic acid concentration in a still-warm muscle, the biophysical properties of meat are altered leading to PSE. A series of studies have demonstrated the relation of fiber type compositions and the rate and extent of p.m. pH decline. Karlsson et al. (1999) stated that high frequency of glycogen depleted in fibers at slaughter, in particular, IIb fibers will have an influence on meat quality. However, Larzul et al. (1997) found no significant correlation between fiber traits and pH_u, although IIb fibers were investigated to contain more glycogen than other types of fibers (Fernandez et al. 1995, Klont et al. 1998). In contrast, Ryu and Kim (2005) and Maltin et al. (1997) mentioned a significant inverse relation between pH_u and IIb fibers as well as a positive link between pH_u and the oxidative capacity and the area of slow fibers. In the context of pH_u values, the view that oxidative fibers are desirable in meat quality was supported by Chang et al. (2003), who demonstrated greater abundance of IIa and IIx fibers with higher pH_u in *psoar* muscle. Given these points, the inconsistency on the effects of fiber composition on pH changes may originally come from different pig

breeds and kinds of muscle used in those studies. Obviously, in an attempt to compare the changes of pH among pig muscles, Lefaucheur (2006) concluded that fiber type composition is far related to the rate of p.m. pH decline but closely associated with the extent of p.m. pH decline with the evidence of decreasing pH_u when the proportion of fast glycolytic fibers increases.

2.5.3 Intramuscular fat

The intramuscular fat is an important characteristic in evaluation of sensory quality. Intramuscular fat (IMF) is composed of two major constituents, triglycerides and phospholipids representing more than 50% of fresh pig *Longissimus* muscle (Faucitano et al. 2004, Leseigneur-Meynier and Gandemer 1991). Despite intensive research, the relationship between histological characteristics and sensory pork quality is still unclear. It was observed that IMF values are closely related to triglyceride content in the muscle, which was in turn negatively associated with mean fiber area (Karlsson et al. 1999) and that neutral lipids are contained in all type I fibers but only in about 26% of IIa and 1% of IIb fibers. In a comparison on muscle fiber characteristics of eight different breeding populations, Maltin et al. (1997) indicated a significant contribution of fast twitch oxidative glycolytic fibers to the variation of meat tenderness. More specifically, Essen-Gustavsson et al. (1994) found lipids present mainly in type I and some IIa fibers, whereas Henckel et al. (1997) reported the frequency of IIb fiber and intramuscular fat content are positively correlated and thus flavor and tenderness seemed to have a negative relationship with IIa but positive correlation with IIb fibers. Interestingly, in a sensory test by taste panels, the meat from half-Chinese crossbred pigs was more tender, juicy and more tasty than that from European pigs, but in a consumer's survey Touraille et al. (1989) found no difference in the overall acceptability between the two pork sources. In contrast to data regarding total IMF content, muscle fiber proportion is also related to the nature of phospholipids, which is shown to present more in oxidative than glycolytic muscles (Leseigneur-Meynier and Gandemer 1991). Because phospholipids are determinants of cooked meat flavor, muscle fiber is likely to have an effect on flavor but further studies are encouraged to unravel this correlation.

2.5.4 Meat tenderness

Meat tenderness is influenced by many factors including the physical size of muscle bundle and the amount of connective tissue and fat. Historically, studies on pork tenderness have received little attention since it was considered to be relatively tender, but in practice this trait varied among muscles and animals (Devol et al. 1988). The extent to which meat tenderizes can be accessed by shear force measurement because of their rather high correlation (Boccard et al. 1981, Hovenier et al. 1993). In pigs, shear force has been detected to have low correlation with muscle fiber percentage (Gentry et al. 2004, Henckel et al. 1997). However, when taking only type I fibers into consideration, both a negative (Chang et al. 2003) and a positive (Ryu and Kim 2005) correlation between this fiber type and shear force of cooked pork LD muscle have been reported. Previous findings also demonstrated that fast glycolytic fibers are negatively related with toughness in pigs (Karlsson et al. 1993) and cattle (O'Halloran et al. 1997). In fact, increasing the proportion of type I fibers was considered to improve tenderness and juiciness in cattle (Maltin et al. 1998). Nevertheless, in normal cattle breeds no correlations were found between fiber characteristics and meat quality traits (Wegner et al. 2000), including tenderness (Vestergaard et al. 2000). Despite variable and sometimes controversial results, there is evidence suggesting the relationship between muscle fiber characteristics and meat tenderness, especially in pork (Klont et al. 1998).

2.5.5 Meat color

Another important quality parameter, lightness, was discovered to be negatively related to type I and IIa fiber percentages, implying that a decrease in these two types would lead to increasing lightness (Ryu and Kim 2005). Similarly, in a F2 population Duroc x Berlin Miniature Pig, it was found that IIb fibers are accompanied with a light color and high conductivity (Fiedler et al. 2003). These two findings are also in agreement with results from a study by Larzul et al. (1997), who demonstrated that lightness (L*) is positively related to the percentage of white fibers and negatively to the percentage of red fibers which are in line with the their color characteristics. Not surprisingly, this may reflect the amount of myoglobin found in the tissues as similar results were

additionally mentioned (Depreux et al. 2002). However, there was no evidence on the relationship between muscle fiber and fiber characteristics with both L* and a* (redness) values (Maltin et al. 1997). The discrepancy on the effect of muscle fiber on L* data between two studies might come from the selection of pigs with the presence of absence of halothane positive (nn) as this genotype offered a significantly higher L* value (Depreux et al. 2002). In short, these findings have evidenced and clarified the effects of muscle fibers on meat color accordingly with the metabolic characteristics of individual fiber.

2.6 Important genes related to growth performance and meat quality traits

By different pathways, a number of genes have been detected to influence on the growth performance and meat quality traits. Some of them have been used either extensively or exclusively in industry (Rothschild 2004). A list of these genes is presented in Table 6.

2.6.1 Genes affecting muscle fibers and growth performance

Muscle fiber formation occurs during embryonic development including two stages of primary and secondary generation (Lefaucheur et al. 1995). During this period, the myogenesis is under control of the MyoD gene family consisting of four structural related genes MYOD1 (MYF3), myogenin (MYOG or MYF4), MYF5 and MRF4 (MYF6) (Olson 1990, Tepas and Visscher 1994). The MYOD1 and MYF5 genes are involved in myoblasts proliferation and they were found to directly affect the proportion of fast-twitch oxidative fibers and the fast-twitch low-oxidative fibers of pigs being crosses of Pietrain x (Polish LW x Polish Landrace) and thereby also influence the metabolic properties of muscle (Kłosowska et al. 2004). However, these authors did not observe any correlation between genotype of MYOG, MYOD1 and MYF5 and fiber diameters as well as the fiber number per unit area. The other gene, MYF4, is expressed in all myoblasts starting from differentiation to cell fusion and also marks the end of the myoblast proliferation. In deed, a significant difference of two homozygous genotypes for birth weight, growth rate and lean weight was reported (te Pas et al. 1999). As explained by these authors, a higher birth weight and more muscle fibers could account

for a higher growth rate and carcass weight at slaughter. Higher lean yield could have arisen from more muscle fibers and thus the myogenin plays a crutial role during the formation of muscle fibers. The expression of the last gene in the MyoD family, MYF6, is also involved in the differentiation and maturation of myotubes and highly expressed postnatally (Wyszynska-Koko and Kurył 2004). However, experimental outcomes demonstrating its effects on muscle fibers are still limited.

The expression of c-fos proto-oncogene (FOS) is also necessary in controlling cell growth and differentiation of embryonal, haematopoietic, excitatory and osteoblastic cells (Reiner et al. 2002). In a F2-crossbred of Meishan x Pietrain, a significcant association between the *B*-allele of this gene and an increase in the total numbers of fibers and white fibers were discovered (Reiner et al. 2002). The fiber proportion and diameters were also unraveled to have a relationship with the FOS genotype supporting the functional role of FOS in myogenesis (Piechaczyk and Blanchard 1994).

2.6.2 Genes affecting muscle fibers and meat quality traits

It is well known that pigs homozygous for the halothane (HAL) sensitivity allele (nn) are highly stress susceptible and often induces accelerated pH decline p.m. leading to a pale, soft and exudative (PSE) meat. Different halothane genotypes have been described to be associated with the content of muscle fibers and thereby affect the meat quality. For instance, Depreux et al. (2002) showed a greater amount of IIb but less type I fibers in pigs carrying the “n” allele (Nn or nn), whereas the NN pigs exhibited higher proportion of IIa/IIx fibers. Moreover, a positive correlation between the relative abundance of IIb fibers and pH_u was observed in pigs free of the allele “n”, but across all genotypes, the relationship between IIb fibers and pH45 was negative. This was curious because, as explained by Depreux et al. (2002), it is opposite with the whole assumption that IIb isoform hydrolyzes ATP rapidly and increases the glycolysis rate. On the other hand, Eggert et al. (2002) suggested that the relative amounts of individual fibers are not attributed by the effects of HAL, or in other words, the process of maturation from one fiber to another is free from the HAL accelerated effect. Obviously, data from various studies uncovered the influence of the HAL gene by

increasing muscle fiber CSA, particularly both the proportion and CSA of glycolytic fibers (Eggert et al. 2002, Essén-Gustavsson et al. 1992, Sellier and Monin 1994).

Table 6: Candidate genes and their observed association with growth and meat quality traits in the pig

Genes	Traits	Industry use[1]	References
A-FABP, H-FABP	Intramuscular fat	-	Gerbens et al. (2001), Zeng et al. (2005)
CAST	Tenderness, muscle fiber	Yes - exclusive	Ciobanu et al. (2004), Kłosowska et al. (2005)
CTSB, CSTB	Backfat, daily gain	-	Russo et al. (2002)
FOS	Muscle fibers	-	Reiner et al. (2002)
GH1	Carcass quality	-	Kurył et al. (2003a), Pierzchała et al. (2004)
HAL (RYR1)	Meat quality/stress	Yes - extensive	Depreux et al. (2002), Essén-Gustavsson et al. (1992)
IGF2	Carcass composition	Yes - exclusive	Chen et al. (2005), Van Laere et al. (2003)
KIT	White color	Yes - exclusive	Giuffra et al. (1999), Pielberg et al. (2002)
LEP	Intake, growth, fat thickness	-	Chen et al. (2004), Křenková et al. (1999)
MC1R	Red/black color	Yes - extensive	Kijas et al. (2001), Kijas et al. (1998)
MC4R	Growth and fatness	Yes - exclusive	Chen et al. (2005), Houston et al. (2004), Kim et al. (2000), Óvilo (2006)
MyoD	Muscle fiber	-	Kłosowska et al. (2004)
PGAM2, PKM2	Glycolytic potential	-	Fontanesi et al. (2003)
PIT1	Growth, carcass traits	-	Brunsch et al. (2002), Pierzchała (2003)
RN, PRKAG3	Meat quality	Yes -extensive/ exclusive	Marinova et al. (1992), Milan et al. (2000)

[1] Rothschild (2004)

Conversely but similar detrimental effect on meat quality has the Rendement Napole (RN) gene, which mainly increases the CSA of red fibers leading to a decrease in relative area of white fibers and thus the fibers are more to oxidative and less to glycolytic metabolism. The RN effect, namely, “acid meat” phenotype indicated a positive correlation between glycolytic potential and lactate content and pH_u (Monin

and Sellier 1985). In fact, results from Marinova et al. (1992) proved that pigs carrying RN^- gene have higher glycogen content in white muscles especially in glycolytic fibers. This was later confirmed by a conclusion that white muscles are more affected than red muscle and that the glycogen content increased in LD muscle (Lebret et al. 1999). Further findings from these authors also emphasized higher enzyme activities and relative area of type II red fibers in the dominant RN^- carriers. A causative mutation (R200Q) for the RN^- gene in the PRKAG3 gene encoding for a muscle-specific isoform of the regulatory γ subunit of adenosine monophosphat-acitvated protein kinase is additionally reported (Milan et al. 2000).

Calpastatin (CAST) is a specific inhibitor of calpain, a Ca^{2+}-activated protease family and is considered to be involved in the initiation of myofibrillar protein degradation in living muscle (Goll et al. 1992). Genes coding for calpastatin and calpain are therefore suggested as candidate genes for growth and meat quality of skeletal muscle. As an illustration, Kurył et al. (2003b) characterized the polymorphism of CAST using three restriction enzymes (*Hinf*I, *Msp*I, *Rsa*I) in several pig breeds and lines and concluded that pigs with different genotypes differ in backfat and weight and that there is an association between eye muscle area and the CAST genotype. This is supported in a preliminary study of Kłosowska et al. (2005) showing that *Longissimus lumborum* diameters of all types of muscle fibers are significantly related to the Stamboek pigs' genotype at locus CAST. The percentage of fast-twitch glycolytic fibers in a bundle was also concluded to change the metabolic properties of muscles and thereby meat quality. Although this locus is located in the 6^{th} intron of the gene, Kłosowska et al. (2005) suggested that this intronic mutation should be considered both as a marker for muscle microstructure characteristics and as the causal mutation itself.

3 Materials and methods

3.1 Materials

In this section, materials used in the study such as animals, all other biological materials, chemicals, kits, reagents, media, softwares, equipments and their sources are mentioned.

3.1.1 Animals

For the identification of muscle fiber types, three pig breeds of Mongcai, Pietrain and Duroc and two crossbreeds of Duroc x Pietrain and Duroc x Berlin Miniature Pig were examined. From the pure Mongcai raised in a state farm in the Central of Vietnam, six finishing pigs with live weight 33.8 ± 6 kg at the age of 215 ± 16 days were collected. Fresh LD muscle samples taken at the 13/14th ribs were stored in liquid nitrogen and further processed for RNA and cDNA synthesis at the advanced lab of Cantho University, Vietnam. All other animals were kept at the research farm Frankenforst, University of Bonn, Germany, where they were performance tested according to the German performance test directives (ZDS, 2003). Of the commercial breeds, Pietrain and Duroc, nine unrelated animals were sampled. In addition, two F2-populations were generated and used in this study: a population based on reciprocal crossbreeding of Duroc and Pietrain (DUPI) and a cross of Duroc and Berlin Miniature pigs (DUMI), which is a cross of Vietnamese Pot Belly Pigs, Saddle Back Pigs and German Landrace (Hardge et al. 1999). Out of 598 F2-DUPI and 420 F2-DUMI, six discordant sib-pairs from each cross, representing extremes for the trait loin eye muscle area (large vs. small) were selected for MyHC quantification. Pigs with large eye muscle area were designated high performing; others were designated low performing.

3.1.2 Materials for laboratory analysis

3.1.2.1 Chemicals, kits, biological and other materials

Amersham Biosciences (Freiburg): GFXTM PCR DNA and Gel Band Purification Kit, poly (dA), Cy3 and Cy5 dyes

Applied Biosystems (Foster City): SYBR® Green Universal PCR Master Mix

Beckman Coulter (Krefeld): CEQ™ 8000 Genetic Analysis System, Dye Terminator Cycle Sequencing (DTCS), Glycogen

Biomol (Hamburg): Phenol, Phenol/Chlorophormllsoamyl alcohol (25:24:1), Lambda DNA Eco9lI (BstE II) and Lambda DNA HindIJJ

Biozym Diagnostic (Hessisch-Oldendorf): Sequagel XR Sequencing Gel (National Diagnostics) and SequiTherm EXCELTM II DNA Sequencing kit-LC (Epicentre Technologies)

Corning (Amsterdam): GAPS II coated slides

DYNAL Biotech (Hamburg): Dynabeads oligo (dT)25

Eppendorf (Hamburg): 2.5x RealMasterMix/ 20x SYBR Solution

Invitrogen Life Technologies (Karlsruhe): DTT, SuperScript^{TM} II RNase H^{-} Reverse Transcriptase, 5 X first strand buffer, Random Primers

MBI Fermentas (St. Leon-Rot): Glycogen

Promega (Mannheim): BSA, pGEM®-T vector, RQ1 RNase-free DNase. RNasin Ribonuclease inhibitor, 2X rapid ligation buffer, T4 DNA ligase, Pronto!^{TM} Plus systems

Qiagen (Hilden): RNeasy® Mini kit, QIAquick PCR Purification Kit, Mini Elute^{TM} Reaction Cleanup Kit

Roth (Karlsruhe): Acetic acid, Agar-Agar, Ampicillin, Bromophenol blue, Dimethyl sulfoxide (DMSO), Ethylenediaminetetraacetic acid (EDTA), Ethanol, Ethidium bromide, Hydrochloric acid, Isopropyl -D-thiogalactoside (IPTG), Kohrso1in FF, Nitric acid, Peptone, Potassium dihydrogen phosphate, 2-Propanol, Silver nitrate, Sodium acetate, Sodium carbonate, Sodium chioride, Sodium hydroxide, Trichloromethane/chiorophorm, Tris, X-Gal (5 -bromo-4-chloro-3-indolylbeta-D-galactopyranoside), Yeast extract

Sigma-Aldrich Chemie GmbH (Munich): Agarose, Ammonium acetate, Calcium chloride, Formaldehyde, GenElute^{TM} Plasmid Miniprep Kit, Glutamine, Hepes, Isopropanol, Magnesium chloride, 2-Mercaptoethanol,

Ohigonucleotide primers, Penicillin, Phenol red solution, 10 X PCR reaction buffer, Potassium chloride, Sodium dodecyl sulfate (SDS), Taq DNA polymerase, yeast tRNA

Stratagene (Amsterdam): 5 c DH *Escherichia coli* competent cells

USB (Ohio): ExoSAP-IT

3.1.2.2 Reagents and media

All solutions used in these investigations were prepared with deionized Millipore water (ddH_2O) and pH was adjusted with sodium hydroxide (NaOH) or hydrochloric acid (HCl).

LB-agar plate	Sodium chloride	8.0 g
	Peptone	8.0 g
	Yeast extract	4.0 g
	Agar-Agar	12.0 g
	Sodium hydroxide (40 mg/ml)	480.0 µl
	ddH_2O added to	800.0 ml
LB-broth	Sodium chloride	8.0 g
	Peptone	8.0 g
	Yeast extract	4.0 g
	Sodium hydroxide (40 mg/ml)	480.0 µl
	ddH_2O added to	800.0 ml
TBE (10x) buffer	Tris	108.0 g
	Boric acid	55.0 g
	EDTA (0.5 M)	40.0 ml
	ddH_2O added to	1000.0 ml
TAE (50x) buffer, pH 8.0	Tris	242.0 mg
	Acetic acid	57.1 ml
	EDTA (0.5 M)	100.0 ml

	ddH_2O added to	1000.0 ml
TE (1x) buffer	Tris (1 M)	10.0 ml
	EDTA (0.5 M)	2.0 ml
	ddH_2O added to	1000.0 ml
X-gal	X-gal	50.0 mg
	N, N'-dimethylformamide	1.0 ml
10x FA buffer, pH 7.0	MOPS	41.8 g
	Sodium acetate	4.1 g
	EDTA (0.5M)	20.0 ml
	ddH_2O added to	1000.0 ml
1.2% FA gel	Agarose	1.2 g
	10 x FA buffer	10.0 ml
	RNase free H_2O	90.0 ml
	Ethidium bromide	2.0 µl
	Formaldehyde (37%)	1.8 ml
Agarose loading buffer	Bromophenol blue	0.0625 g
	Xylencyanol	0.0625 g
	Glycerol	7.5 ml
	ddH_2O added to	25 ml
Digestion buffer	NaCl	100 mM
	Tris-HCl	50 mM
	EDTA pH 8.0	1mM
SDS solution	Sodium dodecylsulfat in ddH_2O	10% (w/v)
Proteinase K solution	Protein K in 1 x TE bufer	2% (w/v)

dNTP solution	dATP (100 mM)	10.0 µl
	dCTP (100 mM)	10.0 µl
	dGTP (100 mM)	10.0 µl
	dTTP (100 mM)	10.0 µl
	ddH_2O added to	400.0 µl
IPTG solution	IPTG	1.2 g
	ddH_2O added to	10.0 µl
3M Sodium Acetate, pH 5.2	Sodium Acetate	123.1 g
	ddH_2O added to	500 ml
1M EDTA, pH 8.0	EDTA	37.3 g
	ddH_2O added to	1000 ml
Phenol Chloroform	Phenol : Chloroform	1 : 1 (v/v)

3.1.3 Used softwares

BLAST	http://www.ncbi.nlm.nih.gov/BLAST/
Multi sequence alignment	http://prodes.toulouse.inra.fr/multalin/multalin.html
Primer3	http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
Restriction enzyme analysis	http://tools.neb.com/NEBcutter2/index.php
Significant Analysis of Microarray	http://www-stat.stanford.edu/~tibs/SAM/
CRI-MAP (version 2.4)	http://compgen.rutgers.edu/multimap/crimap/
SAS (version 8.02)	SAS Institute Inc., NC, USA
GenePix pro 4.0 software	Axon Instruments, Foster city, CA
Image Analysis Program	LI-COR Biotechnology, USA
Primer Express® Software	Applied Biosystems, Foster city, CA, USA

3.1.4 Equipment

ABI PRISM® 7000 SDS	Applied Biosystems, Foster city, USA
Automated sequencer (LI-COR 4200)	MWG Biotech, Ebersberg
Centrifuge	Hermle, Wehingen
CEQ™ 8000 Genetic Analysis System	Beckman Coulter GmbH, Krefeld
Electrophoresis (for agarose gels)	BioRad, Munich
Electrophoresis (vertical apparatus)	Consort, Turnhout
GFL 7601 hybridization chamber	Fisher scientific, Leicestershire, UK
HERA safe Bioflow safety hood	Heraeus Instruments, Meckenheim
Hybridization cassettes	TeleChem International, Sunnyvale
Incubator	Heraeus, Hanau
Millipore apparatus	Millipore corporation, USA
PCR thermocycler (PTC100)	MJ Research, USA & BioRad, Germany
pH meter	Kohermann
Savant SpeedVac®	TeleChem International, Sunnyvale
Power supply PAC 3000	Biorad, Munich
Spectrophotometer, Ultrospec™ 2100 *pro* UV/Visible	Amersham Bioscience, Munich
Thermalshake Gerhardt	John Morris scientific, Melbourne
Tuttnauer autoclave	Connections unlimited, Wettenberg
Ultra low freezer (-80^{o}C)	Labotect GmbH, Gottingen
UV Transilluminator (Uvi-tec)	Uni Equip, Martinsried, Germany
Spectrophotometer (DU-62)	Beckman, Unterschleissheim-Lohhof
GenePix 4000B Microarray Scanner	Axon Instruments, Foster city, CA

3.2 Methods

In this section, the basic molecular genetics methods used in this study are described first, followed by microarray hybridizations and data analysis. Further more, the methodologies used for comparative sequencing, SNP screening and genotyping of candidate genes are given. The overview of the present work is illustrated in Figure 6.

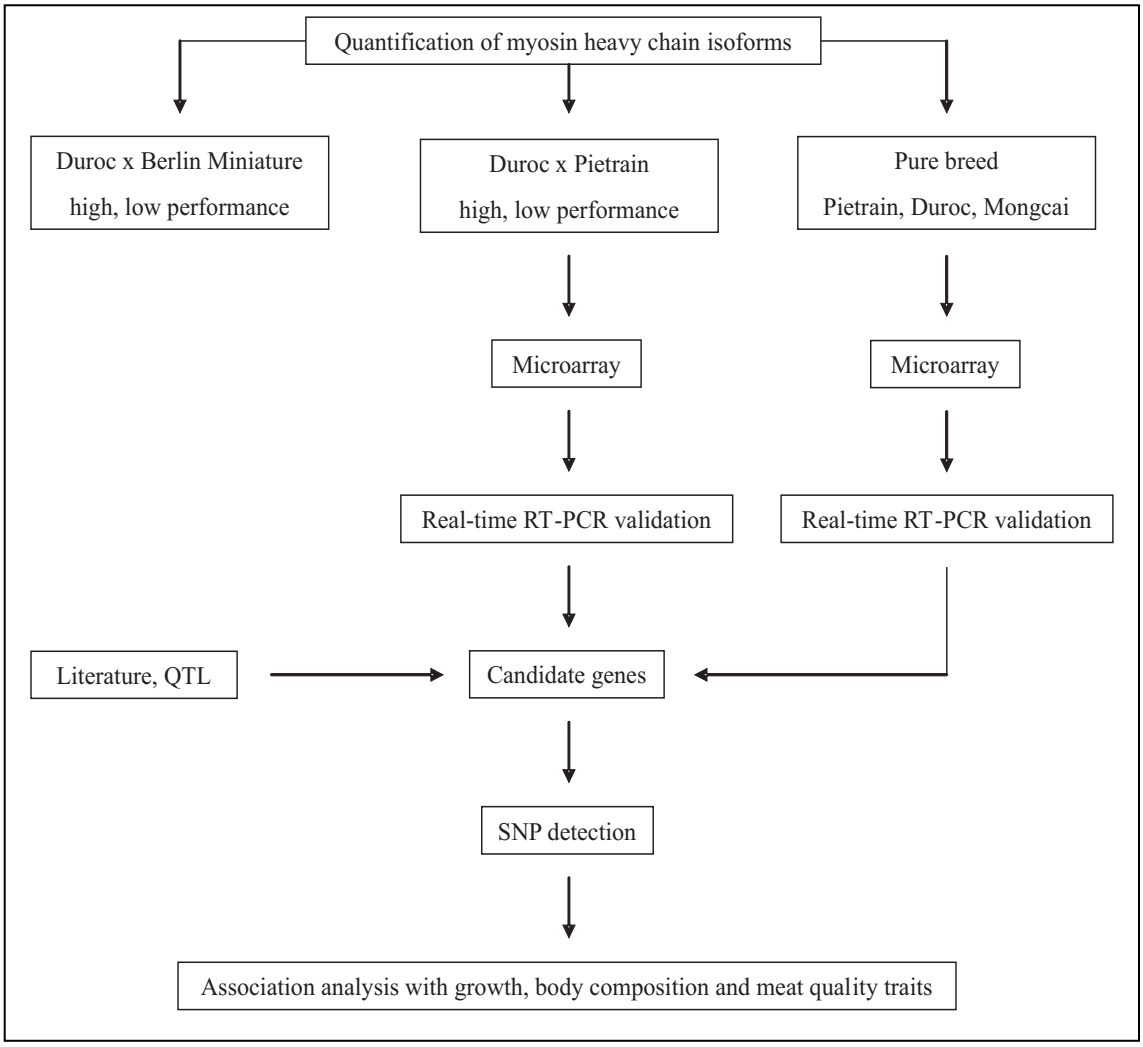


Figure 6: Overview of the present study

3.2.1 RNA isolation of muscle samples

Total RNA was isolated from individual LD skeletal muscle by using TRIzol Reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. In brief, muscle sample was first grinded in a mortar, then mixed and homogenized with 1 ml

TRIzol using syringes and needles. To ensure complete dissociation of nucleoprotein complexes, the sample was allowed to stand for 5 min before adding 0.2 ml of chloroform. The mixture was shaken and left at room temperature for 10 min and centrifuged at 12,000 x g for 15 min at 4^{o}C. The upper aqueous phase was transferred to another fresh centrifuge tube and RNA was precipitated with 0.5 ml of isopropanol. After being incubated at room temperature for 10 min, the sample was centrifuged at 12,000 x g for 10 min at 4^{o}C to get the RNA pellet, which was subsequently washed by 75% (v/v) ethanol. Centrifugation was then performed and the RNA pellet was air-dried and resuspended in 40 µl of DEPC treated water.

In order to remove possible contaminating genomic DNA, the extracted RNA was treated with 5 µl RQ1 DNase buffer, 5 units DNase and 40 units of RNase inhibitor in a 40 µl reaction volume. The mixture was incubated at 37^{o}C for 1h followed by purification with the RNeasy Mini Kit. Concentration of clean-up RNA was determined spectrophotometrically at 260 and 280 nm; the purity of RNA was estimated by the ratio A260/A280 with respect to contaminants that absorb in the UV. Additional examination of integrity was done by denaturing agarose gel electrophoresis and ethilium bromide staining. Finally, the purified RNA was stored at -80^{o}C for further analysis.

3.2.2 Reverse transcription and cDNA synthesis

First-strand cDNA was synthesized from individual RNA using SuperScript II enzyme. The reaction started by adding 1 µl of Oligo $(dT)_{15}$ primer (100 µM) and 1 µl random primer to 1 µg of total RNA for annealing by incubation at 68^{o}C for 5 min followed by cooling on ice for 2 min. A transcription mixture including 4 µl first strand 5X buffer, 1 µl 0.1 DTT, 1 µl dNTP mix (10 mM each), 1 µl (200 units) SuperScript II reverse transcriptase, 1 µl (40 units) of RNasin Ribonuclease inhibitor and RNase-free water was prepared to make a final volume of 20 µl. The reaction was incubated at 25^{o}C for 5 min followed by 42^{o}C for 1h and stopped by heating at 70^{o}C for 15 min. The resulting cDNA was tested with housekeeping gene 18S primers and kept at -20^{o}C until use.

3.2.3 PCR product purification

The PCR product (20 µl) was separated by running in a 1% (w/v) agarose gel containing ethidium bromide (5 µl) in 1x TAE buffer. DNA fragment, which was visualized under an ultraviolet transilluminator, was cut and placed in a fresh tube at -20°C for 30 min before adding 600 µl of 1 x TE buffer to be homogenized. An equal volume (600 µl) of phenol: chloroform (1:1) was added, mixed and vortexed well. After centrifugation at 14,000 g for 15 min at 4°C, the aqueous phase was carefully transferred to a new microcentrifuge tube. Subsequently, to remove the traced phenol, another 600 µl of chloroform was added, shaken vigorously and centrifuged for 10 min. The upper phase was transferred to a new tube and DNA was precipitated by adding double volume of cool absolute ethanol and 1:10 volume of 3 M sodium acetate (pH 5.2) acting as neutralizing the highly charged phosphate backbone and promoting hydrophobic interactions. The solution was mixed gently and stored at -20°C overnight or alternatively at -80° for 2 hours. The DNA was later recovered by centrifugation at 14,000 g for 30 min at 4°C. To remove residual salt in the sample, the DNA pellet was washed by 75% (v/v) ethanol followed by drying at room temperature and resuspending in 7 µl of water. The purified cDNAs were kept at -20°C for ligation.

3.2.4 Ligation

Ligation of a PCR fragment into plasmid pGEM®-T vector was done in a 5 µl reaction mix containing 2.5 µl of 2x ligation buffer, 0.5 µl of pGEM®-T (50 µg/µl), 0.5 µl of T4 DNA ligase (3 units/µl) and 1.5 µl target template. The reaction was incubated at room temperature for 1h or, to get a maximum of transformants, at 4°C overnight.

3.2.5 Transformation

For DNA transformation, the entire ligation reaction was added to an aliquot of 60 µl competent JM109 *E. coli* cells and incubated on ice for 30 min. This mixture was then heat-shocked briefly in a 42°C water bath for 90 sec and immediately returned to ice for

2 min. Next, an addition of 600 µl nutrient medium (LB-broth) was performed and all were incubated at 37°C for 90 min in a shaker. At the same time, ampicillin treated LB-agar (50 mg/L LB-agar) plates including 20 µl of X-Gal (50 mg/ml in N, N'-dimethyl-formamide) and 20 µl of IPTG were prepared. At the end of incubation period, the transformation culture was plated on two previously prepared LB-agar plates and incubated at 37°C overnight.

3.2.6 Colony screening and plasmid DNA isolation

Colonies were screened based on the activity of β-galactosidase as white and blue for the presence and absence of insert DNA fragments. This happened because β-galactosidase, a product of the *lacZ* gene in pGEM®-T vector, interacts with IPTG to produce a blue color. On the other hand, when an insert was successfully ligated, the *lacZ* gene is disrupted; β-galactosidase is not produced resulting in white color in the colonies. Two white colony representatives from each plate were picked up and suspended in 30 µl 1x buffer for M13 PCR for further confirmation of transformation. The same colonies were cultured in 600 µl ampicillin/LB-broth (5 mg / 100 ml) in a shaking incubator at 37°C for further plasmid isolation. In addition, to be used as a control for later comparison of the length of amplified DNA fragments, a blue colony was also selected from any plate.

Plasmid DNA was isolated by using GenElute™ Plasmid Miniprep Kit followed the manufacturer's instructions. Briefly, 5 ml of bacterial culture were centrifuged at 14,000 g for 1 min for harvesting cells, the supernatant was discarded. These cells were resuspended and vortexed in 200 µl of resuspension solution before adding 200 µl of lysis solution to lyse the solution. The mixture was subsequently mixed by inversion of tubes until it became clear and viscous. After incubating at room temperature for 4 min, cell precipitation was done by adding 350 µl of neutralization/binding buffer, mixed gently and centrifuged at 14000 g for 10 min. At the same time, the GeneElute Miniprep column was prepared by adding 500 µl of preparation solution, centrifuging shortly and discarding the flow-through. After that, the clear supernatant was transferred to this binding column and centrifuged at 14000 g for 1 min. The flow-through was discarded and the column was washed by adding 750 µl of wash solution followed by

centrifugation at 14000 g for 1 min. To elute DNA, the column was transferred to a fresh collection tube; 50 µl of ddH_2O was added and centrifuged at 14000 g for 1 min. The column was discarded and the DNA plasmid was then collected.

For determination of plasmid size and quality, 5 µl of plasmid together with 2 µl loading buffer was checked by agarose gel electrophoresis. In addition, the quantity of plasmid was also measured by reading the absorbance at 260 nm in a spectrophotometer UV/visible light (Beckman Du® 62). An aliquot of DNA plasmid was subjected to sequence check; the rest was stored at -20°C to be used as template for setting up the standard curve in real-time PCR.

3.2.7 M13 PCR and sequencing for product confirmation

M13 PCR was done to confirm the insertion of the fragment into the plasmid. Bacterial suspensions were boiled at 95°C for 15 min and these lysed bacterial solutions were used as templates. The M13 PCR was carried out in a 20 µl reaction including 1 µl 10x PCR buffer, 10 µl lysed bacterial solution, 0.5 µl dNTP (10 mM), 0.5 µl (10 µM) of each M13 primer (forward: 5'-TTGTAAAACGACGGCCAGT-3'; reverse: 5'-CAGGAAACAGCTATGACC-3') and 0.1 Unit of Taq polymerase. The PCR reaction was performed with a thermal cycling program of 95°C for 5 min followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec, 70°C for 1 min and an additional extension step for 5 min at 70°C. An aliquot of 5 µl PCR product was then electrophoresed in 2% (w/v) agarose gel with 0.8 µg/ml ethidium bromide in 1 x TAE buffer. Under UV-transilluminator, length differentiation of PCR fragments was identified. The M13 PCR products from white colonies were selected for subsequent sequencing while bacterial cultures of these colonies were expanded in a volume of 5 ml and incubated at 37°C overnight in a shaking incubator for plasmid isolation.

The M13 products were used as templates for sequencing according to the dideoxy chain-termination method using SequiTherm Excel™ II DNA Sequencing Kit (Epicentre Technologies, Biozym). For each PCR fragment, four sequencing reactions were prepared with different termination mix solutions namely deaza-dATP, ddCTP, ddTTP, ddGTP in a total volume of 3 µl containing 1 µl of termination mix and 2 µl of premix solution, which included of 3.5 µl of 3.5X sequencing buffer, 0.25 µl of 700-

IRD labeled primer SP6 (10 µM): 5'-TAAATCCACTGTGATATCTTATG-3', 0.25 µl of 800-IRD labeled primer T7 (10 µM): 5'-ATTATGCTGAGTGATATCCCGCT-3', 5 units of SequiTherm Excel II DNA polymerase and 3.9 µl PCR products. These sequencing reactions were carried out at 95°C for 3 min followed by 29 cycles of 95°C for 15 sec, 59 °C for 15 sec, and 70°C for 1 min and the reactions were ended by adding 1.5 µl of stopping buffer. The sequence reaction products were then denatured at 95°C for 5 min and immediately kept on ice and loaded on a 6% Sequagel XR sequencing gel, 41 cm in length (National Diagnostics, Biozym). Electrophoresis was performed overnight (or at least 6h) in 1 x TBE buffer at 50°C, 50 W and 1200 V in the LI-COR 4200 automated DNA sequencer. The gel image was later analyzed by Image Analysis Program version 4.10 (LI-COR Biotechnology).

The results from sequence analysis were compared with published sequences using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Plasmids from those clones with identity percentage higher than 90% were considered to be significant similarities and were isolated for downstream application.

3.2.8 Real-time RT-PCR

Real-time RT-PCR was carried out in an ABI Prism® 7000 SDS instrument based on the changes in fluorescence proportional to the increase of product. SYBR® Green, which emits a fluorescent signal upon binding to double stranded DNA, was used as a detector. Fluorescence values were recorded during every cycle representing the amount of product amplified to a point known as threshold cycle (Ct). The higher the initial transcript amount, the sooner accumulated product was detected in the PCR process.

The plasmid serial dilution was prepared by converting concentration of plasmid (ng/µl) into numbers of molecules using the website http://molbiol.ru/eng/scripts/01_07.html. The plasmid concentration was diluted several folds from 10^1 to 10^8 so that the concentration would cover the range of target concentration in the muscle samples. A further step was to start the PCR assay to test whether a suitable standard curve could be achieved for high PCR efficiency.

Prior to quantification, the optimum primer concentration was obtained by trying different combinations from 200 nM to 600 nM. Results from these primer

combinations were compared and the one with lowest threshold cycle and minimizing non-specific amplification was selected for subsequent reaction. After selection of primer concentration, a final assay consisted of 2 µl cDNA as template, up and down stream primers and SYBR Green Universal PCR Master Mix containing SYBR Green I Dye, AmpliTaq Gold DNA polymerase, dNTPs with dUTP, passive reference and optimized buffer components were performed in a total volume of 20 µl reaction. Thermal parameters used to amplify the template started with an initial denaturation at 95^{o}C for 10 min followed by 40 cycles of 95^{o}C for 15s denaturation and 60^{o}C for 1 min annealing and extension. A dissociation curve was generated at the end of the last cycle by collecting the fluorescence data at 60^{o}C and taking measurements every 7s until the temperature reached 95^{o}C. Final quantification analysis was done by amplifying serial dilutions of target plasmid DNA. The concentration of unknown cDNA was calculated according to the standard curve, and expression level of transcripts was described relatively to the transcript of 18S gene that was found to be stable between the samples of equal amount of analyzed cDNA. Table 7 presents the list of primers used in real-time RT-PCR for MyHC quantification.

Table 7: List of primers used to quantify MyHC isoforms

Gene	Primer sequence (5' – 3')		Annealing Temp. (^{o}C)	Length (bp)	Accession no.
MyHC I	Fw:	AAGGGCTTGAACGAGGAGTAGA	60	177	AB053226
	Rev:	TTATTCTGCTTCCTCCAAAGGG			
MyHC IIa	Fw:	GCTGAGCGAGCTGAAATCC	60	137	AB025260
	Rev:	ACTGAGACACCAGAGCTTCT			
MyHC IIx	Fw:	AGAAGATCAACTGAGTGAACT	60	149	AB025262
	Rev:	AGAGCTGAGAAACTAACGTG			
MyHC IIb	Fw:	ATGAAGAGGAACCACATTA	57	166	AB025261
	Rev:	TTATTGCCTCAGTAGCTTG			
18S	Fw:	GAGCGAAAGCATTTGCCAAG	60	101	AF102857
	Rev:	GGCATCGTTTATGGTCGGAAC			

3.3 Histological analysis

In order to differentiate the three main fiber types, samples were stained using the myosin adenosine triphosphatase (mATPase) method modified from Brooke and Kaiser (1970). In brief, crossed-sectional samples of 10 µm were cut from the LD muscle in a cryostat at -20^{o}C. These sections were stained for mATPase activity after both acid (pH = 4.6 and 4.3) and alkaline (pH = 9.4) preincubation. The samples were examined by an image analysis system, which could result in differentiation into type I (slow, red muscle, oxidative), type IIa (fast, red muscle, oxidative) and type IIb/IIx (fast, white muscle, glycolytic). In total, 15 samples taken from F2 animals of the DUPI population were used and 400 fibers were counted per sample.

3.4 Microarray hybridization techniques and data analysis

3.4.1 Home-made microarray construction

A description of the constructed microarrays used in this experiment is given in Ponsuksili et al. (2005). In brief, immobilized probes were amplified from pooled cDNAs derived from liver RNA of five breeds (Duroc, Hampshire, Pietrain, German Landrace and F2-DUMI animals based on the cross of Duroc and Berlin Miniature Pig). These cDNA were derived from three sources: (1) ESTs corresponding to genes resulted from differential display band patterns when comparing expression in liver and other nine organs; (2) ESTs diet associated expressed in liver and intestine identified by differential display and (3) amplified probes from genes encoding proteins involved in hepatic metabolic pathways. According to the requirements of the spotting service provider (Genescan Europe, Freiburg, Germany), a volume of 200 µl PCR products were purified, precipitated and finally resuspended in 20 µl of spotting buffer. In total, 238 mRNA transcripts involved in lipid, carbohydrate, and nitrogen metabolism as well as plasma proteins and lipoproteins for binding and transportation of metabolites and hormones were spotted on glass slides in triplicate.

The other pig microarray (QIAGEN array) was distributed by the U.S. Pig Genome Coordination Program. This array was produced with the QIAGEN Array-Ready Oligo

Set for the Pig Genome (version 1.0) and the Pig Genome Oligo Extension Set (version 1.0), in which 13,297 70-mer probes were spotted representing porcine gene sequences with a hit to human, mouse, or pig gene transcript. Details on the gene lists, data sheets and product profiles are available from http://www.animalgenome.org/pigs/resources/array_request.html.

3.4.2 cDNA labeling and purification

The protocol used for the home-made and QIAGEN array experiments was similar. However, in the home-made array, muscles from four discordant sib-pairs of the F2-DUPI resource population were selected based on their differences in loin eye muscle area; while in the QIAGEN array, the targets were muscles from six un-related Pietrain and six un-related Mongcai pigs.

Total RNAs isolated from skeletal muscle were used for labeling. To minimize genetic variability among individual pigs, the RNAs in each group were pooled for labeling and hybridization according to the manufacture's protocol (Pronto!^{TM} Plus System, Promega). A 20 µl labeling reaction consisted of 5 µg total RNA, 3 µg of random primer and 2 µg of oligo (dT) primer and nuclease-free water. The RNA/primer solution was mixed gently and incubated at 70^{o}C for 10 min, and placed on ice. While incubating, two labeling mixes with either Cy3 or Cy5 dye were prepared. In both tubes, a total volume of 20 µl was performed with equal amount of ChipShot^{TM} reverse transcriptase 5x reaction buffer (8 µl) and $MgCl_2$ (25mM, 4.8 µl) and ChipShot^{TM} reverse transcriptase (3.2 µl). However, only 2 µl of dNTP mix and 1 µl of Cy3-dCTP (1mM) were added to one tube instead of 3 µl of dNTP mix and 1 µl of Cy5-dCTP (1mM) added to another. When the incubation reaction was ready, the entire 20 µl of labeling mix was transferred accordingly to each tube of RNA/primer solution, mixed well and centrifuged briefly before incubating at room temperature (22 – 25^{o}C) for 10 min. After that, the reactions were incubated at 42^{o}C for 2 hours. As soon as the dyes were added, the reactions were always protected from light. At the end of the incubation step, a stop solution including 1.0 µl of RNase H and 0.35 µl of RNase solution was added to each cDNA-synthesis reaction followed by incubating at 37^{o}C for 15 min. The Cy$^{®}$-label cDNAs were continuously purified with the ChipShot^{TM} Labeling Clean-up

System (Pronto!^{TM} Plus System, Promega) followed the provided protocol. The samples were finally eluted with 60 µl of elution buffer and the cDNAs were accessed to quantitative absorbance at 260, 550 and 650 nm for calculation of frequency of dye incorporation and stored at -20^{o}C until hybridization.

3.4.3 Hybridization and post-hybridization wash of cDNA microarrays

Before hybridization, array slides were treated in pre-soak and pre-hybridization solution followed the manufacture'a protocol (Pronto!^{TM} Plus System, Promega). In the final step, the slides were dipped in nuclease-free water and dried by short centrifugation. When the activated slides were ready, an appropriate amount of each dye-labeled cDNA was dried using a speed vaccum concentrator. Then, the pellets were dissolved in the required volume of Pronto!^{TM} universal hybridization solution. This mixture was then incubated at 95^{o}C for 5 min to denature the probes, centrifuged at 13,5000 x g for 2 min to collect condensation and transferred to the surface of the printed arrays. Slides were covered by glass cover slips (BDH Co.), placed in the hybridization cassette (TeleChem) and fixed well. The chamber-array assembly was incubated in a hybridization oven at 42^{o}C for 14-20 hours. At the end of hybridization, slides were washed with washing solution supplied in the Pronto!^{TM} Plus System Kit, shortly centrifuged and scanned immediately.

3.4.4 Image capture and data analysis

Images were obtained from GenePix 4000B Microarray Scanner (Axon Instruments) and analyzed using GenPix Pro 6.0 software. Normalization was performed for each image so that the mean of the ratio of medians of the entire features was equal to 1. In order to overcome the effect of non-uniform noise emanating from spotted DNA as well as to ensure that the signal level was sufficiently high above the background, only genes with signal to noise ratio greater than 3 were selected for further analysis. The statistical test for different expression was done by Excel-based Significance Analysis of Microarray (SAM) software (http://www-stat.stanford.edu/%7Etibs/SAM).

3.4.5 Validation of microarray data results by real-time RT-PCR

The real-time RT-PCR procedures were prepared and assayed as described in section 3.2.8. List of primers for validation and gene expression is shown in Table 8.

Table 8: List of primers used in real-time RT-PCR for microarray data validation and gene expression

Gene	Primer sequence (5' – 3')		Annealing Temp. (^{o}C)	Length (bp)	Accession no.
FHL1C					AJ275967
	Fw :	GGAGGACTTCTACTGCGTGACT	55	153	
	Rev:	GCCAGCTTCTTAGAGCAGGTAA			
FTH1[1]					D15071
	Fw :	TAAGCTGGCCTCCCGGAGAC	55	130	
	Rev:	GGTACACTAAGGAAAGAACT			
RAMP1					NM_214199
	Fw :	CTTCAGAAACTGCCCCGTCT	60 → 55	387	
	Rev:	CAAGAGGTTGGACGGAACTG			
CKM					AY754869
	Fw 1:	GCTCGTCGGAGGTAGAACAG	55	276	
	Rev 1:	ATTGGCTGGAACTCTGGTTG			
TNNC1					AY958072
	Fw 1:	GCATGAAGGATGACAGCAAA	55	124	
	Rev 1:	CCTGTCGCCTGAAGCATTAT			

[1]Rattink et al. (2001a)

3.5 SNP detection and association analysis

3.5.1 DNA extraction

Hundred mg of sample tissue was cut into small pieces and placed in a 1.5 ml tube, after that 700 µl of digestion buffer, 70 µl of 10% SDS and 18 µl of proteinase K for protein digestion was added. The mixture was incubated overnight at 37^{o}C in a shaker at 90 rpm. Completely digested tissue resulted in a viscous homogeneous solution. To extract

DNA, 700 µl of phenol-chloroform was added into each tube and gently mix by several inversions until an emulsion formed. The mixture was separated into 3 phases after centrifugation at 10,000 rpm for 10 min, a lower phenol-chloroform phase, an interphase of precipitated protein and an upper phase containing DNA. The aqueous phase was transferred to another 2 ml tube followed by an addition of 700 µl chloroform and centrifugation at 10,000 rpm for 10 min. For DNA precipitation, the DNA phase was transferred to a fresh tube and mixed with 700 µl of isopropanol and 70 µl of sodium acetate. After centrifuging at 10,000 rpm for 5 min, a DNA pellet was collected and the supernatant was removed. The pellet was washed with 200 µl of 70% ethanol for the removal of excess salt and left air dry after centrifugation. In the final step, the pellet was dissolved in 200-500 µl of 1x TA buffer. DNA concentration and integrity was evaluated by a spectrophotometer. The working solution of DNA was prepared by diluting stock DNA in 1x TA buffer to the concentration of 50 ng/µl. Stock DNA solution was stored at -20^{o}C and the working solution was kept at 4^{o}C.

3.5.2 Comparative sequencing for SNP detection

Prior to the sequencing procedure, PCR with specific primer was performed and 5 µl of the PCR product was checked on 2% agarose gel. A sharp band visualized under UV/ transilluminator showed primer specifity and the PCR products were ready to be cleaned up. A mixture of 1 µl of ExoSAP-IT with 5 µl of PCR product was incubated at 37^{o}C for 30 min followed by ExoSAP-IT inactivation at 80^{o}C for 15 min. To the clean PCR product subjected to sequencing PCR, 2 µl of either forward or reverse primer, 4 µl DTCS master mix (DNA polymerase, pyrophosphatase, buffer, dNTPs, and dye terminators) and 8 µl of ddH_2O were added. The sample was programmed for 30 cycles started by denaturation at 96^{o}C for 20 sec followed by annealing at 50^{o}C for 20 sec and extension at 60^{o}C for 4 min. Just before the reaction was finished, prepared a stop solution including 2 µl of 3M Sodium Acetate (pH 5.2), 2 µl of 100 mM Na_2-EDTA (pH 8.0) and 1 µl of glycogen (20 mg/ml) was prepared. To each of the labeled tubes, 5 µl of the stop solution and 60 µl cold 95% ethanol was added, mixed thoroughly and immediately centrifuged at 14,000 rpm at 4^{o}C for 15 min. The supernatant was removed

and the pellet was rinsed two times with 200 µl cold 70 % ethanol. For each rinse, a centrifugation was immediately applied at 14,000 rpm at 4°C for 5 min. After that, the supernatant was removed and the pellet was air dried (or vacuum dried) for 10 min. Finally, the pellet was resuspended with 40 µl of sample loading solution and was ready for sequencing. For preparation of loading samples into the instrument, the resuspended samples were transferred to the wells of the sample plate and overlaid with one drop of light mineral oil, at the mean time, the separation buffer was prepared in another plate and both plates were loaded into the instrument and started the desired method in the CEQ™ 8000 Genetic Analysis System.

3.5.3 Genotyping

Primers used for comparative sequencing to detect SNP and PCR-RFLP for genotyping are presented in Table 9. DNA samples from the DUPI resource population were genotyped by using PCR-RFLP (Restriction Fragment Length Polymorphim). The restriction enzymes were selected according to their recognition (http://tools.neb.com/NEBcutter2/index.php) of the polymorphic sites. First, the fragment covers the SNP were amplified with a specific primer. Next, a digestion reaction containing 1 unit of enzyme, 1 µl of 10x restriction buffer and 1 µg of DNA was incubated at 37°C overnight for complete digestion. The digested product was checked by electrophoresis on 3% aragose gel, 90 V for 1 hour. Different fragment length between non- and digested DNAs reflected the genotype of a specific sample.

3.6 Linkage mapping

Two point and multiple procedures of the CRI-MAP package version 2.4 were used for linkage mapping (Green et al. 1990).

3.7 Statistical analysis

The SAS package version 8.02 (SAS Inc., Cary, NC, USA) was applied for statistical analyses. Means of the relative abundance of mRNA level of the four MYH isoforms were compared using a *t*-test. The significance of differences of MYH isoforms in different breeds was evaluated by the general linear model (GLM), in which breed was used as a fixed effect. Overall correlation coefficients between two methods in muscle typing were tested using partial correlation coefficients CORR procedure.

Analysis of variance using PROC MIXED of SAS was performed to investigate the effect of genotypes on body composition and meat quality traits. All factors found to affect the phenotypes were included: genotype, sex, boar, and parity as fixed effects, sow as random factor and carcass weight as covariate. Mean separation for significant difference ($P < 0.05$) was accomplished by the PDIFF option of the least square procedure.

Table 9: List of primers used for comparative sequencing and genotyping

Gene	Primer sequence (5' – 3')		Annealing Temp.(°C)	Length (bp)	Accession No.
FHL1C	Fw 1:	CGGTACAGGGATCACTTTCTCT	55	485	NM_214375
	Rev 1:	GTGTTGGCGCAGAACTTGT			
	Fw 2:	GCTGCAGGGAAAGAAGTACG	55	638	
	Rev 2:	TCTTGCATCCAGCACACTTC			
	Fw 3:	CGCTGTGGAGGACCAGTATT	55	837	
	Rev 3:	AGGGGAAAGGTCGGTTAGAA			
	Fw 4:[a]	CGTCACAGCGAATACTTCT	55	537	
	Rev 4:[a]	CGGCTTCTCTAACCACTCC			
FHL1C	Fw 5:	GTCTCCAGCCATCGCATG	56	610	AJ275967
	Rev 5:	TCGCAACCTTAATCCCACTC			
FTH1[b]	Fw:	TAAGCTGGCCTCCCGGAGAC	55	130	D15071
	Rev:	GGTACACTAAGGAAAGAACT			
TNNC1	Fw 2:	GAGGCTCTGTTGCTGTTTCC	56	449	AY958072
	Rev 2:	GAGCAGCTGTCCATGTCAGA			
CKM	Fw 2:	CGACTTATGGCACGACTTCA	56	443	DQ153192
	Rev 2:	AACTCGGGTCAGAGCACAG			

[a] Davoli et al. (2002) [b] Rattink et al. (2001a)

4 Results

4.1 Transcript abundance of MyHC isoforms

The F2 generations of DUPI and DUMI largely vary in many traits especially those related to body composition and muscularity. In order to address the relationship between traits related to muscularity and relative abundance of MyHC isoforms, divergent sib-pairs of two F2 resource populations based on divergent founder breeds were used. The selected animals for expression profile of four MyHC isoforms were similar in birth weight, live weight and slaughter weight assuring that the difference in the traits of interest between them was not due to the different stages of development. Loin eye muscle area was the only trait that was statistically different ($P < 0.01$) between two groups in both the DUPI and the DUMI population. The mean phenotypic values of all animals together with 6 sib-pairs are shown in Table 10.

Table 10: Performance of animals from F2 resource population: mean ± standard deviation of 598 DUPI, 420 DUMI and selected small/large eye muscle area pigs

Item		Large	Small muscle	All
DUPI				
	Number of animals	6	6	598
	Birth weight (kg)	1.7 ± 0.3	1.6 ± 0.3	1.5 ± 0.3
	Live weight (kg)	109 ± 4.8	111.1 ± 4.9	110.9 ± 6.2
	Slaughter weight (kg)	84.8 ± 4.6	84.1 ± 4.7	85.2 ± 5.0
	Age (days)	181 ± 13	176 ± 23	178 ± 16
	Eye muscle area (cm^2)	60.3^a ± 3.7	43.3^b ± 3.6	51.2 ± 6.1
DUMI				
	Number of animals	6	6	420
	Birth weight (kg)	0.9 ± 0.3	0.7 ± 0.2	0.9 ± 0.3
	Live weight (kg)	90.3 ± 12.6	79.7 ± 16	87.0 ± 13.2
	Slaughter weight (kg)	69.1 ± 9.4	61.6 ± 13.4	67.4 ± 10.5
	Age (days)	203 ± 2	201 ± 2	201 ± 3
	Eye muscle area (cm^2)	28.5^a ± 4.3	17.7^b ± 1.5	24.5 ± 4.5

[a, b] ($P < 0.001$)

4.1.1 MyHC composition in different breeds determined by real-time RT-PCR

In this study, the relative standard curve method was applied to quantify the relative gene expression of MyHC slow/I, IIa, IIx, IIb and 18S. The standard curve gradients of all genes had similar slopes (3.75 ± 0.04) implying similar PCR amplification efficiency (Figure 7).

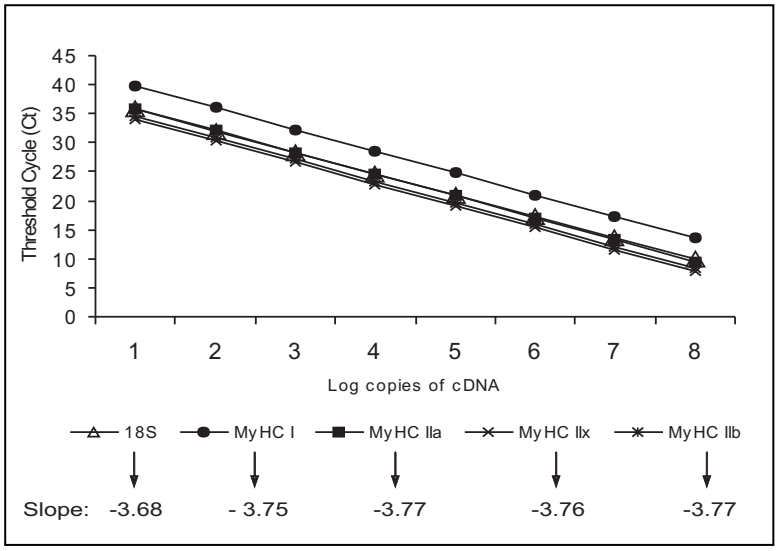


Figure 7: Relative standard curves of five genes 18S, MyHC slow/I, MyHC IIa, MyHC IIx and MyHC IIb. Similar slope values suggest the similarity in amplification efficiency

Animals of two commercial breeds and a native breed were examined to be different in traits related to muscularity and meat quality. In both commercial breeds Duroc and Pietrain, abundance of MyHC type IIb accounted for more than half of the MyHC transcripts (59.7 and 65.4%, relatively). The proportion of MyHC type IIb transcripts was similar in the crossbred pigs DUPI (61.3%) and DUMI (54.0%). In terms of slow fiber, DUMI had a significantly higher proportion of MyHC I isoform than other breeds, with the exception of Mongcai (Table 11). The native pig, Mongcai, showed very low MyHC IIb transcript abundance compared to the other breeds and crosses. However, the Mongcai showed much higher relative abundance of the other MyHC isoforms. Therefore, in the case of MyHC IIa and IIb the difference in fiber composition among breeds was due to the Mongcai pigs.

Table 11: Proportion (%) of MyHC in different breeds quantified by real-time RT-PCR (least squares means ± standard errors)

Trait	Breed					*P*-value
	Pietrain	Duroc	Mongcai	DUPI	DUMI	
MyHC I	9.4 ± 4.0[b]	10.0 ± 5.7[b]	24.1 ± 4.0[ac]	15.0 ± 2.8[bc]	25.9 ± 2.8[a]	<0.01
MyHC IIa	4.3 ± 2.5[b]	12.2 ± 3.5[b]	28.6 ± 2.5[a]	8.8 ± 1.7[b]	7.4 ± 1.7[b]	<0.001
MyHC IIx	20.9 ± 3.0[b]	18.1 ± 4.3[bc]	35.9 ± 3.0[a]	14.9 ± 2.1[bc]	12.7 ± 2.1[c]	<0.001
MyHC IIb	65.4 ± 4.8[a]	59.7 ± 6.7[a]	11.4 ± 4.8[b]	61.3 ± 3.4[a]	54.0 ± 3.4[a]	<0.001

[a, b, c] Means in the same row with different superscripts differ, $P < 0.05$

4.1.2 MyHC composition in F2-DUPI determined by mATPase staining

By ATPase staining after acidic preincubation (pH 4.6) three fiber types were distinguished, i.e. black type I, white or unstained type IIa and gray type IIb/IIx fibers (Figure 8), whereas ATPase staining after preincubation at pH 4.3 and pH 9.4 just allowed to discriminate type I and type II fibers. In the same samples by real-time RT-PCR with specific primers for four adult MyHC isoforms, the relative abundance of the transcripts was determined. The correlations between these phenotypes ranged between 0.53 and 0.72 and were highly significant ($P < 0.05$) for the corresponding pairs of muscle fibers characterized by histochemistry and transcript abundances of the MyHC isoforms (Table 12).

Table 12: Muscle fiber typing (%) by ATPase staining and quantitative RT-PCR assays (means ± standard deviation)

Trait	ATPase pH4.3	ATPase pH9.4	ATPase pH4.6	Real-time RT-PCR	Coefficient of correlation*
Type I	18.4 ± 6.6	17.3 ± 6.1			
Type II	81.6 ± 6.6	82.7 ± 6.1			
MyHC I			16.1 ± 7.1	18.3 ± 6.8	0.72 ($P = 0.004$)
MyHC IIa			3.5 ± 2.4	12.8 ± 11.1	0.67 ($P = 0.009$)
MyHC IIx			80.4 ± 7.2[**]	16.5 ± 9.9	0.53 ($P = 0.05$)[**]
MyHC IIb				52.4 ± 18.6	

* Coefficient of correlation was calculated based on the ATPase pH4.6 and real-time RT-PCR values

[**] These figures were evaluated for both MyHC IIx and MyHC IIb isoforms

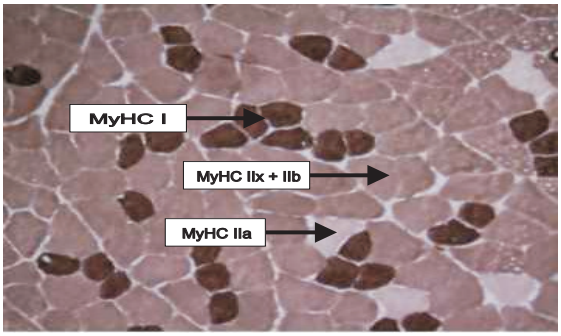


Figure 8: Muscle fiber typing detected by mATPase staining after acidic pre-incubation at pH 4.6. Black, white and gray color represent type I, type IIa and type IIx/IIb fiber, respectively

4.1.3 MyHC proportion in large and small eye muscle area within breed

Owing to genetic characteristics, a larger percentage of muscle area from DUPI than DUMI was observed. However, this did not affect the shift of direction of fiber type composition, as seen in Figure 9 and Figure 10. The discordant sib-pairs in the DUPI and the DUMI population differed significantly ($P < 0.05$) in the relative abundance of type IIb transcripts. In the DUPI resource population, similar transcript level of MyHC I was found in both large and small muscles (14.7 and 15.2%), whereas in DUMI animals these figures were 18.4 and 33.5%, respectively, and thus different at $P < 0.05$. The extreme groups in the DUPI tended to differ in MyHC IIa and IIx transcripts.

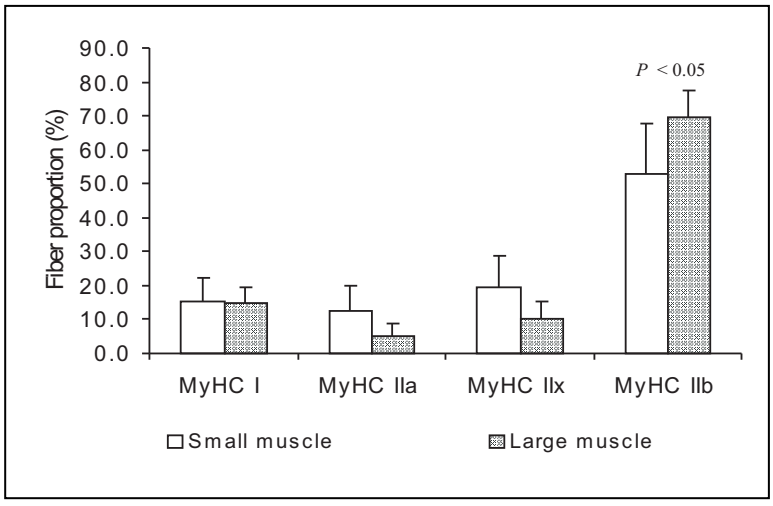


Figure 9: Relative expression of four MyHC isoforms of small and large LD muscle from DUPI resource population

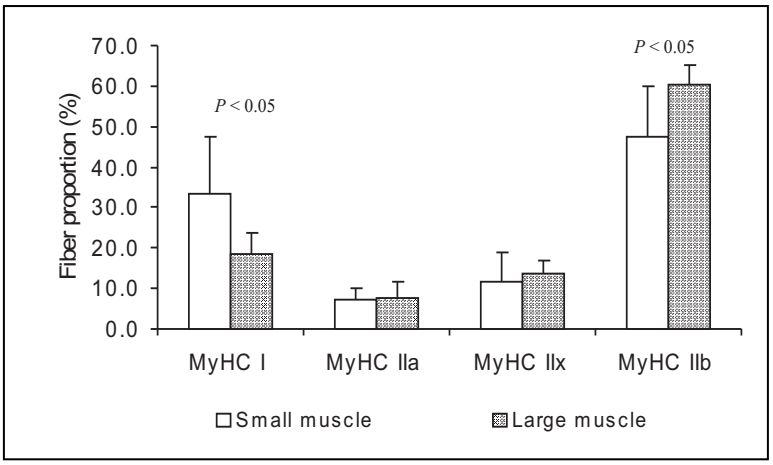


Figure 10: Relative expression of four MyHC isoforms of small and large LD muscle from DUMI resource population

4.2 Identification of candidate genes for body composition and meat quality

4.2.1 Microarray experiment

Average phenotypic values of loin eye muscle area (cm^2) of all 598 animals together as well as 4 small and 4 large muscle pigs used in this study were 51.2 ± 6.1, 41.7 ± 2.6 and 60 ± 2.1, respectively.

To overcome dye effects between slides, a total of 4 microarrays were hybridized in two replicates and dye swap. After normalization, the data were further screened according to the signal to noise ratio (SNR). Those with SNR larger than 3 were included to create a list of 81 genes that were ready for statistical analysis. In order to identify differentially expressed genes, the SAM software was applied and the up and down-regulated genes were selected based on the list of significant genes with the lowest False Discovery Rate q-values < 0.01. Finally, three genes were shown to be highly expressed in the small muscle animal group (Table 13).

Table 13: List of genes highly expressed in animals with small eye muscle area

Accession No.	Gene	Function
D15071	FTH1 *(Ferritin heavy-chain)*	iron storage
AJ275967	FHL1C *(Four-and-a-half LIM domain 1 protein, isoform C)*	muscle development
AF312385	RAMP1 *(Receptor activity modifying protein 1)*	cellular communication

4.2.2 Validation of microarray results by real-time RT-PCR

In the home-made microarray experiment, four discordant sib-pairs of F2-DUPI animals were used. The animals were selected with the standard that age, slaughter weight and carcass weight were not significantly different between two extreme groups. Thus, differences between small or large muscle areas were genetically originated. For validation by real-time RT-PCR, these animals were included together with four additional, independent sib-pairs to strengthen the data analysis. Table 14 summarizes traits selected for the microarray experiment and real-time assay.

Table 14: Descriptions of F2-DUPI resource population used for microarray and real-time RT-PCR (means ± standard deviation)

Trait	Pig eye muscle area		*P*-value
	Small (n = 4)	Large (n = 4)	
Microarray study			
Age (day)	188.0 ± 35.0	177.7 ± 14.7	n.s
Slaughter weight (kg)	112.5 ± 6.5	111.3 ± 7.3	n.s
Carcass weight (kg)	84.8 ± 8.1	84.7 ± 8.0	n.s
Eye muscle area (cm^2)	41.7 ± 2.6	60.0 ± 2.1	< 0.001
Real-time validation (additional)			
Age (day)	175.5 ± 11.0	179.5 ± 7.4	n.s
Slaughter weight (kg)	110.5 ± 3.8	111.3 ± 1.0	n.s
Carcass weight (kg)	86.1 ± 3.1	86.7 ± 1.3	n.s
Eye muscle area (cm^2)	44.7 ± 2.2	56.9 ± 2.8	< 0.001

n.s: non significant

4.2.2.1 FTH1 (Ferritin heavy-chain)

The relative mRNA expression of FTH1 gene is shown in Figure 11. In all animals, expression of mRNA were higher in pigs having small eye muscle area, as a result average values for two extreme group were statistically different ($P < 0.05$). This is in line with the microarray output, in which the fold change between two groups was 1.47. It can be stated that, FTH1 is an up-regulated gene in pigs with small muscle area.

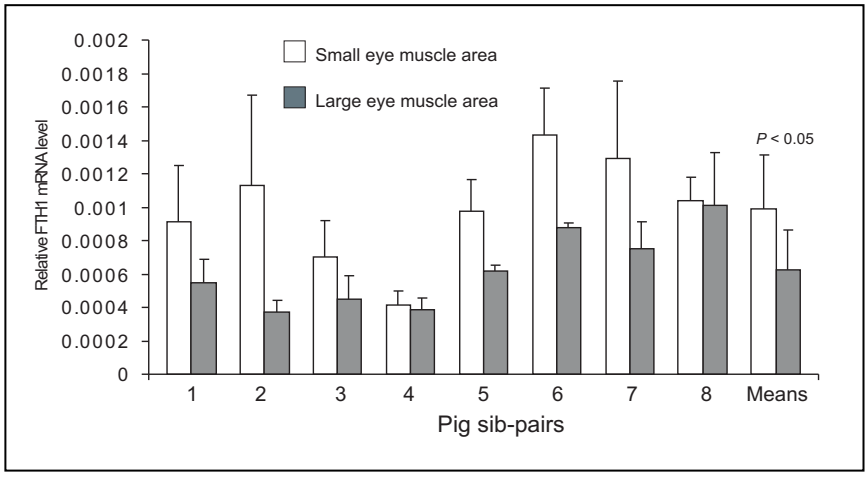


Figure 11: Validation by quantitative real-time RT-PCR, FTH1 is highly expressed in pigs with small compared to large eye muscle area

4.2.2.2 FHL1C (Four-and-a-half LIM domain 1 protein, isoform C)

In small eye muscle area animals the mRNA was highly expressed in most sib-pairs with an exception of one pair, where the expression level was similar (Figure 12). However, between two extreme groups, there was a significant difference ($P < 0.05$) which is in accordance with fold change value from microarray data (1.6 vs. 2.2). In other words, two methods were consistent in identification FHL1C gene as an up-regulated gene in pigs having a small muscle area.

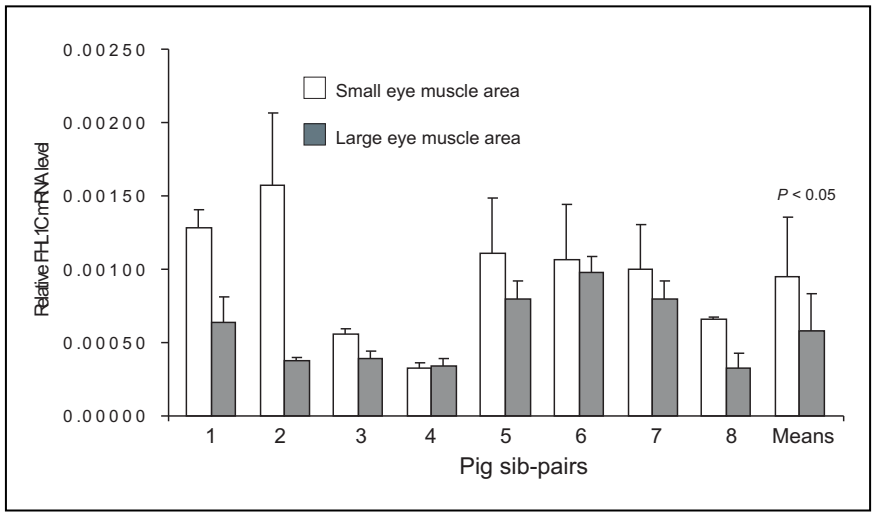


Figure 12: Validation by quantitative real-time RT-PCR, FHL1C is highly expressed in pigs with small compared to large eye muscle area

4.2.2.3 RAMP1 (Receptor activity-modifying protein 1)

The expression of RAMP1 largely varied among sib-pairs as shown in Figure 13. Transcript abundances of small and large eye muscle pigs in pair 1, 3 and 6 were contradictory to those from microarray leading to a non-significant difference in fold change between two experimental groups ($P > 0.05$). In this case, the result from microarray was not confirmed by real-time RT-PCR and hence the proposal that this gene is up-regulated in small loin eye pigs is rejected.

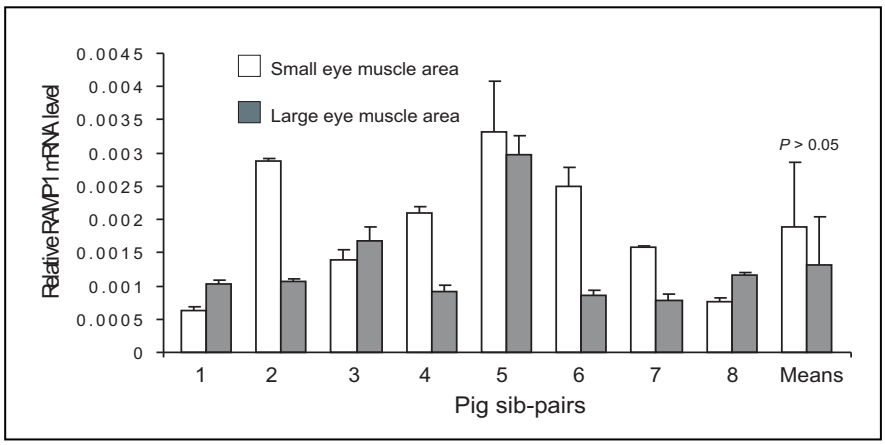


Figure 13: Relative mRNA expression, RAMP1 is not confirmed by quantitative real-time RT-PCR

4.2.3 Relative expression of TNNC1 and CKM in Pietrain and Mongcai

4.2.3.1 TNNC1 (Troponin C, type 1, slow)

In the QIAGEN array, LD muscle from the commercial breed Pietrain and the Vietnamese local breed Mongcai was used to identify gene expression. However, only one hybridization was performed due to the shortage of Mongcai's RNA samples. From the list of differentially expressed genes in this preliminary experiment (data not shown), the TNNC1 was selected by its fold change difference of 1.8. The expression levels of RNAs among the pigs were in a large range but followed the trend that TNNC1 is highly expressed ($P < 0.01$) in Pietrain compared to Mongcai (Figure 14). As a result, an average fold change value of 6.0 was generated and therefore TNNC1 is concluded as an up-regulated gene in Pietrain.

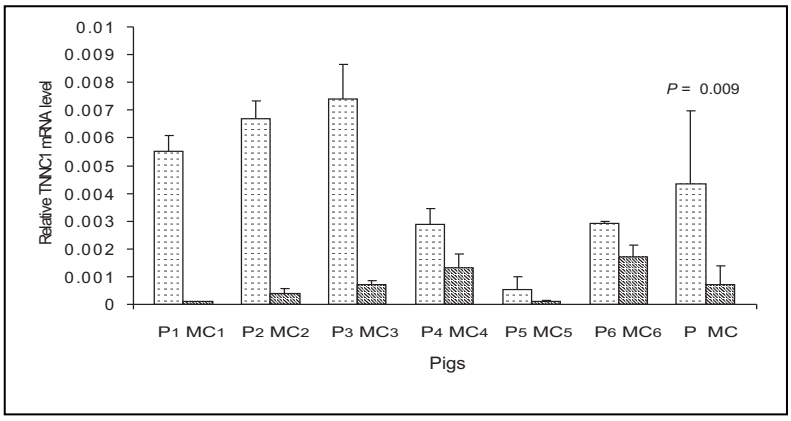


Figure 14: Validation by quantitative real-time RT-PCR of Qiagen array, TNNC1 is highly expressed Pietrain (P) compared to Mongcai (MC)

4.2.3.2 CKM (Creatine kinase, mucle)

In addition to selecting genes derived from microarray results as candidate genes, a literature search was also done and the CKM was chosen based on its positional and proposed functional relationship to skeletal muscle in pigs. Therefore, it was expected that this gene would express differently in commercial and traditional breeds. By real-time RT-PCR, the CKM was found to have lower transcript abundance in Mongcai compared to that of Pietrain (Figure 15). The significant ratio ($P < 0.001$) in relative mRNA level of CKM between the two extreme pig breeds suggests its potential role in muscle and meat quality.

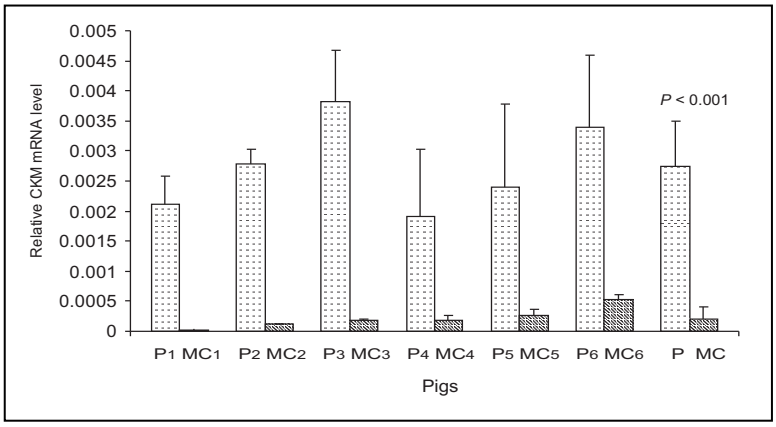


Figure 15: Relative mRNA expression of CKM, transcript abundances in LD muscle of Pietrain (P) are significantly higher than those from Mongcai (MC)

4.2.4 Comparison of expression levels obtained from microarray data and real-time RT-PCR

Figure 16 displays a comparison between results obtained from microarray and those from quantitative real-time RT-PCR. In general, the two methods are consistent in the expression level of RNA transcripts with the exception of TNNC1, where real-time RT-PCR was more sensitive than microarray with the fold change difference 6.0 vs. 1.8.

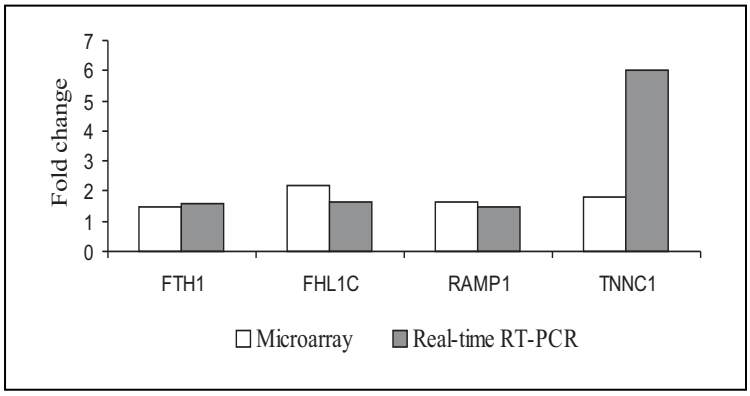


Figure 16: Fold change comparison between results obtained from real-time RT-PCR and microarray experiments

4.3 SNP detection and genotyping

For all genes, SNPs were detected in the F2-DUPI resource population. SNP locations and restriction enzymes used for PCR-RFLP are displayed in Table 15. In detail, a 130-bp product of FTH1 was amplified and two SNPs were discovered in this 3'UTR at position 698 (T → C) and 714 (G → A). Two restriction enzymes *Msp*I and *Bbu*I were used for genotyping. The *Msp*I digested products had 94, 31 and 5 bp and the *Bbu*I produced fragment sizes of 78 and 52 bp. The other gene derived from microarray, FHL1C did not show any SNP in all exons and some intron regions. For TNNC1, by comparative sequencing in the intron part of the gene with the accession No. AY958072, four SNPs were detected at position 629 (A → G); 657 (T → C); 716 (C → G) and 769 (C → G). Out of these SNPs, the C → G SNP at position 716 (Figure 17) was selected for genotyping in the amplified product of 449 bp. The restriction enzyme *Ava*II separated the PCR product into two fragments of 337 and 112 bp (Figure 18).

Finally, an amplification of 443 bp for the CKM gene was performed and one SNP (G → A) was found in the promoter region at the 354 position. The polymorphic site was cut by the *Hae*II restriction enzyme producing fragment sizes of 350 and 93 bp.

Table 15: Detected SNPs, location and restriction enzymes used for PCR-RFLP

Gene accession No.	Gene symbol	SNP, location	Enzyme	Recognition site
D15071	FTH1	T/C, 698	*Msp*I	5'…C↓CGG…3'
D15071	FTH1	G/A, 714	*Bbu*I	5'…GCATG↓C…3'
AY958072	TNNC1	C/G, 716	*Ava*II	5'…G↓GWCC…3'
DQ153192	CKM	G/A, 354	*Hae*II	5'…RGCGC↓Y…3'

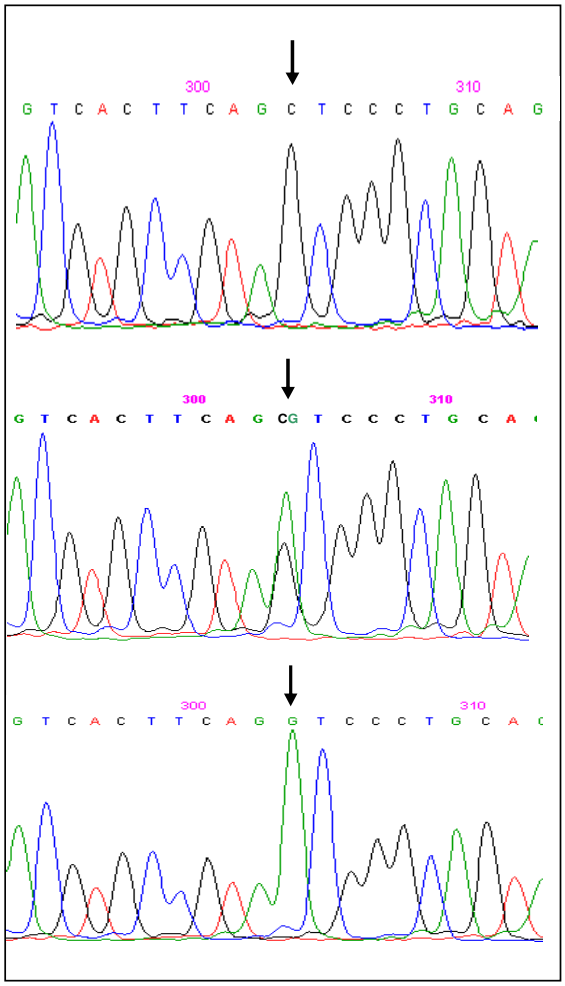


Figure 17: A representative image showing SNP by comparative sequencing. Arrows show SNP site. Top pig in homozygous for C, middle pig is heterozygote with both alleles C and G, bottom pig is homozygous for G.

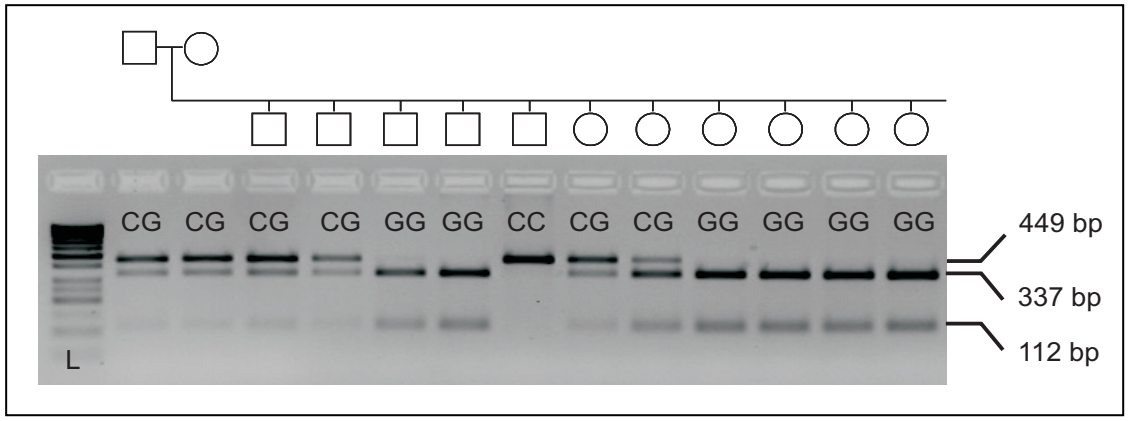


Figure 18: PCR-RFLP for genotyping TNNC1 in F2-DUPI resource population. Electrophoresis of the digested PCR product was performed in a 2.5% gel containing ethidium bromide and visualized by a UV transiluminator

L: O'RangerRuler 50 bp DNA Ladder, MBI Fermentas

4.4 Association analysis of candidate genes with performance traits

4.4.1 Descriptive statistics of traits analyzed

Number of observations, means and standard deviation of traits from animals used for the association study are listed in Table 16 for FTH1, TNNC1 and CKM. With regards to the variation of traits among genotypes it can be mentioned that in almost all cases the variation was not over 30% with the exception of drip loss percentage and conductivity values C24S in all genes. Body weight at birth and weaning stage varied larger than at later stages and thus leading to higher variation of ADG1 and ADG2 in comparison with ADG3 or overall daily gain.

Table 16: Descriptive statistics of traits analyzed (means ± SD)

Trait	FTH1 (n = 337)	TNNC1 (n = 287)	CKM (n = 335)
Body weight (kg)			
Birth	1.52 ± 0.33	1.52 ± 0.33	1.52 ± 0.33
Weaning	8.17 ± 2.05	8.24 ± 2.08	8.15 ± 2.05
Test start	24.3 ± 4.7	24.5 ± 4.7	28.3 ± 3.9
Slaughter	111.2 ± 5.9	110.9 ± 5.9	111.2 ± 5.9
Age at test start (day)	70.6 ± 5.2	70.7 ± 5.3	70.6 ± 5.2
Age at slaughter (day)	179.6 ± 16.2	179.8 ± 16.5	179.7 ± 16.2
Average daily gain, ADG (g/day)			
ADG 1	235 ± 61	237 ± 62	235 ± 61
ADG 2	323 ± 62	325 ± 64	323 ± 63
ADG 3	807 ± 91	802 ± 90	806 ± 91
ADG 4	615 ± 55	613 ± 55	614 ± 54
Feed conversion ratio (kg)	2.60 ± 0.25	2.61 ± 0.25	2.60 ± 0.25
Body composition			
Carcass length (cm)	98.2 ± 2.6	98.1 ± 2.6	98.2 ± 2.6
Eye muscle area (cm^2)	51.2 ± 5.4	51.1 ± 5.5	51.2 ± 5.4
Fat area (cm^2)	16.0 ± 2.9	15.9 ± 2.9	15.9 ± 2.9
Fat : meat ratio	0.31 ± 0.06	0.31 ± 0.06	0.31 ± 0.07
Average backfat thickness (mm)	2.1 ± 0.3	2.1 ± 0.3	2.1 ± 0.3
Backfat depth at neck region (mm)	3.4 ± 0.4	3.4 ± 0.4	3.4 ± 0.4
Fat depth at rib 13/14 (mm)	2.7 ± 0.6	2.7 ± 0.6	2.7 ± 0.7
Fat thickness above loin area (mm)	1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.3
Auto FOM lean content	63.8 ± 4.4	63.6 ± 4.5	63.8 ± 4.4
Loin eye area (kg)	6.3 ± 0.6	6.2 ± 0.6	6.3 ± 0.6
Dressing percentage	76.7 ± 1.9	76.7 ± 1.9	76.7 ± 1.9
Estimated lean content of carcass	58.8 ± 2.5	58.8 ± 2.5	58.8 ± 2.5
Meat quality traits			
MC_{Opto} (%)	68.8 ± 5.5	68.9 ± 5.7	68.8 ± 5.5
Shear force (N)	35.2 ± 6.6	35.2 ± 6.9	35.2 ± 6.6
Drip loss (%)	2.1 ± 1.0	2.1 ± 1.0	2.1 ± 1.0
Cooking loss (%)	24.9 ± 2.2	24.7 ± 2.3	24.9 ± 2.2
$pH1_{LD}$	6.58 ± 0.21	6.57 ± 0.21	6.58 ± 0.21
$pH24_{LD}$	5.51 ± 0.09	5.51 ± 0.09	5.51 ± 0.09
$pH24_S$	5.65 ± 0.14	5.65 ± 0.15	5.65 ± 0.14
$C1_{LD}$ (mS/cm)	4.36 ± 0.62	4.37 ± 0.62	4.36 ± 0.61
$C24_{LD}$ (mS/cm)	2.76 ± 0.83	2.78 ± 0.84	2.76 ± 0.83
$C24_S$ (mS/cm)	4.92 ± 2.09	4.98 ± 2.15	4.92 ± 2.09

4.4.2 Association analysis of FTH1

4.4.2.1 Allele and genotype frequency of two SNPs

In both SNPs, the frequencies of the allele were almost equally distributed (47% vs. 53% for SNP1; 52% vs. 48% for SNP2). However, the range in genotype frequencies was large changing from 24% to 45% (SNP1) and 25% to 47% (SNP2). In both cases, the appearance of heterozygous genotypes were more frequent (>45%) than those from the homozygous animals (Table 17).

Table 17: Allele and genotypes frequency of FTH1 gene (SNP1 and SNP2) in the F2-DUPI resource population

	No of pigs	Allele frequency		Genotype frequency		
		T	C	T/T	T/C	C/C
SNP1	335	0.47	0.53	0.24	0.45	0.31
		G	A	G/G	G/A	A/A
SNP2	334	0.52	0.48	0.28	0.47	0.25

4.4.2.2 Effects of FTH1 - SNP1 on performance traits

Table 18 represents the effects of SNP1 on the phenotypes of pigs. The different genotypes of this SNP had no effect on traits analyzed. In fact, there were differences in body weight at any stage of growth between two pig groups containing either homozygous C/C or T/T. However, the differences did not reach a statistic level ($P > 0.05$). Similarly, results showed that overall average daily gain is independent of genotypes although there seemed to be a tendency of fast growing in T/T pigs compared with C/C pigs (608 g vs. 595 g). Also, this SNP did not show any effects on body composition and meat quality traits ($P > 0.05$).

Table 18: Effects of FTH1 - SNP1 genotype on growth, body composition and meat quality traits (least squares means ± standard error)

Trait	Genotype			*P*-Value
	C/C	T/C	T/T	
Body weight (kg)				
Birth	1.49 ± 0.06	1.52 ± 0.06	1.56 ± 0.07	0.659
Weaning	8.89 ± 0.40	8.81 ± 0.37	8.95 ± 0.41	0.952
Test start	25.4 ± 1.0	26.2 ± 0.9	27.2 ± 1.0	0.416
Slaughter	110.8 ± 0.4	110.9 ± 0.3	110.4 ± 0.4	0.879
Age at test start (day)	72.2 ± 1.0	72.0 ± 1.0	72.3 ± 1.0	0.902
Age at slaughter (days)	185.5 ± 2.4	183.2 ± 2.2	180.3 ± 2.5	0.449
Average daily gain, ADG (g/day)				
ADG 1	244 ± 11	248 ± 11	250 ± 12	0.832
ADG 2	333 ± 13	344 ± 12	356 ± 13	0.346
ADG 3	768 ± 15	774 ± 13	781 ± 15	0.849
ADG 4	595 ± 8	602 ± 7	608 ± 8	0.510
FCR from 30 to 105 kg	2.66 ± 0.04	2.69 ± 0.03	2.64 ± 0.04	0.671
Body composition				
Carcass length (cm)	97.7 ± 0.4	98.0 ± 0.3	98.1 ± 0.4	0.550
Eye muscle area (cm^2)	51.0 ± 0.8	51.3 ± 0.7	51.3 ± 0.8	0.857
Fat area (cm^2)	15.9 ± 0.5	15.7 ± 0.4	15.5 ± 0.5	0.883
Fat : meat ratio	0.32 ± 0.01	0.31 ± 0.01	0.31 ± 0.01	0.847
Meat quality traits				
MC_{Opto} (%)	69.4 ± 0.8	68.6 ± 0.7	70.2 ± 0.9	0.587
Shear force (N)	34.0 ± 1.3	34.9 ± 1.2	36.6 ± 1.3	0.595
Drip loss (%)	2.0 ± 0.2	2.1 ± 0.1	2.1 ± 0.2	0.747
Thaw loss (%)	8.3 ± 0.3	7.9 ± 0.2	8.2 ± 0.3	0.433
Cooking loss (%)	24.9 ± 0.5	24.8 ± 0.4	24.7 ± 0.5	0.892
$pH1_{LD}$	6.56 ± 0.04	6.54 ± 0.03	6.53 ± 0.04	0.601
$pH24_{LD}$	5.50 ± 0.01	5.49 ± 0.01	5.50 ± 0.01	0.863
$pH24_{S}$	5.63 ± 0.02	5.60 ± 0.02	5.64 ± 0.02	0.434
$C1_{LD}$ (mS/cm)	4.3 ± 0.1	4.4 ± 0.1	4.4 ± 0.1	0.471
$C24_{LD}$ (mS/cm)	3.1 ± 0.2	2.9 ± 0.2	3.0 ± 0.2	0.578
$C24_{S}$ (mS/cm)	4.9 ± 0.4	4.8 ± 0.4	5.1 ± 0.4	0.960

4.4.2.3 Effects of FTH1 - SNP2 on performance traits

The effects of FTH1 - SNP2 genotypes on phenotypes are presented in Table 19.

Table 19: Effects of FTH1 - SNP2 genotype on growth, body composition and meat quality traits (least squares means ± standard error)

Trait	Genotype frequency			*P*-Value
	A/A	G/A	G/G	
Body weight (kg)				
Birth	1.46 ± 0.07	1.52 ± 0.06	1.55 ± 0.07	0.227
Weaning	8.60 ± 0.43	8.91 ± 0.37	9.00 ± 0.41	0.437
Test start	24.8 ± 1.0^{b}	26.3 ± 0.9^{ab}	27.3 ± 1.0^{a}	0.008
Slaughter	110.8 ± 0.4	110.8 ± 0.3	110.6 ± 0.4	0.680
Age at test start (day)	72.2 ± 1.1	72.1 ± 1.0	72.3 ± 1.0	0.770
Age at slaughter (day)	187.5 ± 2.7^{a}	183.0 ± 2.2^{ab}	180.0 ± 2.5^{b}	0.007
Average daily gain, ADG (g/day)				
ADG 1	238 ± 12	249 ± 10	251 ± 12	0.352
ADG 2	324 ± 13^{b}	345 ± 11^{ab}	357 ± 13^{a}	0.010
ADG 3	759 ± 16	774 ± 13	787 ± 15	0.114
ADG 4	589 ± 9^{b}	602 ± 4^{ab}	611 ± 8^{a}	0.025
FCR from 30 to 105 kg	2.67 ± 0.04	2.69 ± 0.03	2.63 ± 0.04	0.173
Body composition				
Carcass length (cm)	97.7 ± 0.4	97.9 ± 0.3	98.1 ± 0.4	0.640
Eye muscle area (cm^2)	50.8 ± 0.9	51.3 ± 0.7	51.2 ± 0.8	0.704
Fat area (cm^2)	15.9 ± 0.5	15.8 ± 0.4	15.5 ± 0.5	0.490
Fat : meat ratio	0.32 ± 0.01	0.31 ± 0.01	0.31 ± 0.01	0.480
Meat quality traits				
MC_{Opto} (%)	69.2 ± 1.0	69.1 ± 0.7	69.3 ± 0.9	0.951
Shear force (N)	33.9 ± 1.4^{b}	34.8 ± 1.2^{b}	36.8 ± 1.3^{a}	0.025
Drip loss (%)	2.1 ± 0.2	2.1 ± 0.1	2.1 ± 0.2	0.954
Thaw loss (%)	8.4 ± 0.3	8.0 ± 0.2	8.2 ± 0.3	0.344
Cooking loss (%)	24.9 ± 0.5	24.7 ± 0.4	24.7 ± 0.5	0.894
$pH1_{LD}$	6.57 ± 0.04	6.53 ± 0.03	6.54 ± 0.04	0.488
$pH24_{LD}$	5.50 ± 0.01	5.50 ± 0.01	5.50 ± 0.01	0.995
$pH24_{S}$	5.62 ± 0.02	5.62 ± 0.02	5.63 ± 0.02	1.000
$C1_{LD}$ (mS/cm)	4.36 ± 0.11	4.38 ± 0.08	4.40 ± 0.10	0.982
$C24_{LD}$ (mS/cm)	3.00 ± 0.19	2.99 ± 0.17	3.04 ± 0.19	0.988

[a, b] Means in the same row with different supersripts differ, $P < 0.05$

Among parameters for body weight, SNP2 was associated (P = 0.008) with animal weight at the start of the experiment (72 days) but no association was found between different genotypes and birth, weaning or slaughter weight. Moreover, this SNP had an effect on the growing stage when piglets were weaned until they were allocated in the test box (P = 0.01). Although there was no significant difference between genotypes and growth rate in the third stage (from test start to slaughter), the overall daily gain of animals bearing genotype G/G was significantly higher (P = 0.025) than those with genotype A/A (611 g vs. 589 g, respectively). In addition, results of daily gain of the whole period in heterozygous pigs were not different from either of the homozygous animals. The other data collected for association analysis was the body composition. Carcass length, eye muscle area and fat area were almost similar in three genotype groups meaning that different genotypes did not have any effect on these traits. Finally, results showed that shear force is statistically related to the genotypes, especially with homozygous pigs (P = 0.025). Lower shear force was found in animals with A/A genotype (33.9 N) and higher shear force was seen in G/G pigs (36.8). On the other hand, no other significant associations between different genotypes and other meat quality traits such as drip loss, pH and conductivity were observed.

4.4.2.4 Effects of FTH1 haplotype on performance traits

When combining two the SNPs to construct a haplotype, some traits were found to have a significant relation with certain genotypes (Table 20). For example, the age of pigs at slaughter was significantly associated with haplotype GT/GT and AC/AC (P = 0.018). Haplotype AC/AC and CG/GT also effected ADG3 and ADG4 but not the early stages of development (P = 0.014). High daily gain, especially in the last period of growth made haplotype CG/GT the lowest value in terms of FCR (2.52 kg) with the statistic level P = 0.026. Moreover, no association of body composition traits with any SNP has resulted in non-significant difference among pigs bearing different genotypes (P > 0.05). In addition, meat color was found to be associated with haplotypes (P = 0.026), in which the GT/GT haplotype offered higher value than the CG/GT haplotype (70.7 and 65.8, respectively). Different haplotypes were also in association with pH24 value in *Semimembranosus* muscle (P = 0.014) with lowest pH in animals with CG/GT

haplotype (5.55) and highest pH value in the GT/GT haplotype (5.64). Other meat quality traits including shear force, drip loss and conductivity were not influenced by any haplotype.

Table 20: Effects of FTH1 haplotype on growth, body composition and meat quality traits (least squares means ± standard error)

Trait	Haplotype					*P*-Value
	GT/GT	GT/AC	CG/AC	AC/AC	CG/GT	
Body weight (kg)						
Birth	1.55 ± 0.06	1.54 ± 0.06	1.55 ± 0.09	1.49 ± 0.07	1.51 ± 0.10	0.782
Weaning	8.67 ± 0.46	8.61 ± 0.42	9.70 ± 5.9	8.52 ± 0.46	8.85 ± 0.65	0.248
Test start	26.4 ± 0.1	25.4 ± 0.1	26.6 ± 1.3	24.4 ± 1.0	26.5 ± 1.5	0.115
Slaughter	110.5 ± 0.4	110.9 ± 0.4	110.6 ± 0.6	110.3 ± 0.4	111.5 ± 0.7	0.270
Age at test start	72.1 ± 1.7	71.3 ± 1.7	71.6 ± 1.8	71.3 ± 1.7	71.3 ± 1.8	0.256
Age at slaughter (day)	179.7 ± 3.2^{b}	182.6 ± 2.9^{ab}	176.7 ± 4.2^{b}	186.6 ± 3.2^{a}	176.4 ± 4.6^{b}	0.018
Average daily gain, ADG (g/day)						
ADG 1	245 ± 13	247 ± 12	265 ± 17	240 ± 13	251 ± 19	0.636
ADG 2	346 ± 13	335 ± 11	351 ± 17	321 ± 13	352 ± 19	0.126
ADG 3	789 ± 16^{a}	776 ± 15^{ab}	808 ± 22^{a}	757 ± 16^{b}	817 ± 25^{a}	0.037
ADG 4	610 ± 10^{ab}	603 ± 9^{b}	620 ± 13^{ab}	588 ± 10^{c}	628 ± 15^{a}	0.014
FCR (kg)	2.62 ± 0.05^{bc}	2.69 ± 0.04^{a}	2.54 ± 0.07^{b}	2.69 ± 0.05^{ac}	2.52 ± 0.08^{b}	0.026
Body composition						
Carcass length (cm)	98.2 ± 0.4	98.1 ± 0.4	97.8 ± 0.6	97.4 ± 0.4	98.8 ± 0.7	0.170
Eye muscle area (cm^2)	50.7 ± 0.8	50.6 ± 0.7	51.3 ± 1.2	50.0 ± 0.8	50.5 ± 1.3	0.827
Fat area (cm^2)	15.7 ± 0.5	15.8 ± 0.5	15.8 ± 0.7	15.9 ± 0.5	15.4 ± 0.8	0.942
Fat : meat ratio	0.31 ± 0.01	0.32 ± 0.01	0.31 ± 0.02	0.32 ± 0.01	0.31 ± 0.02	0.910
Meat quality traits						
MC_{Opto} (%)	70.7 ± 0.8^{a}	69.5 ± 0.7^{a}	70.4 ± 1.4^{a}	69.0 ± 0.8^{a}	65.8 ± 1.5^{b}	0.026
Shear force (N)	36.0 ± 1.3	34.1 ± 1.1	34.6 ± 1.8	33.3 ± 1.3	37.0 ± 2.0	0.106
Drip loss (%)	2.1 ± 0.2	2.2 ± 0.1	2.0 ± 0.3	2.2 ± 0.2	2.2 ± 0.3	0.854
Thaw loss (%)	8.2 ± 0.3	8.1 ± 0.2	8.1 ± 0.5	8.5 ± 0.3	8.3 ± 0.6	0.538
Cooking loss (%)	24.7 ± 0.4	24.8 ± 0.4	25.1 ± 0.6	25.1 ± 0.4	25.3 ± 0.7	0.737
$pH1_{LD}$	6.53 ± 0.04	6.54 ± 0.04	6.60 ± 0.06	6.55 ± 0.04	6.66 ± 0.06	0.261
$pH24_{LD}$	5.49 ± 0.02	5.49 ± 0.01	5.50 ± 0.02	5.47 ± 0.02	5.46 ± 0.03	0.516
$pH24_{S}$	5.64 ± 0.03^{a}	5.60 ± 0.03^{bc}	5.67 ± 0.04^{ab}	5.58 ± 0.03^{c}	5.55 ± 0.04^{c}	0.014
$C1_{LD}$ (mS/cm)	4.4 ± 0.1	4.4 ± 0.1	4.2 ± 0.2	4.4 ± 0.1	4.4 ± 0.2	0.654
$C24_{LD}$ (mS/cm)	3.0 ± 0.2	2.9 ± 0.2	3.1 ± 0.3	2.9 ± 0.2	2.8 ± 0.3	0.765
$C24_{S}$ (mS/cm)	5.0 ± 0.4	4.7 ± 0.4	5.0 ± 0.6	4.6 ± 0.4	4.8 ± 0.7	0.815

[a, b] Means in the same row with different supersripts differ, $P < 0.05$

4.4.3 Association analysis of TNNC1

4.4.3.1 Allele and genotype frequency of the TNNC1 polymorphic site

In Table 21 the allele and genotype frequency of TNNC1 SNP is shown. The presence of allele "G" was twice as frequent as allele "C" in the polymorphic site of TNNC1 gene (67% vs. 33%). Moreover, the frequency of genotype C/C was very low (8%) compared to the other genotypes C/G (50%) and G/G (42%).

Table 21: Allele and genotype frequency of TNNC1 gene in the F2-DUPI resource population

No of pigs	Allele frequency		Genotype frequency		
	C	G	C/C	C/G	G/G
287	0.33	0.67	0.08	0.50	0.42

4.4.3.2 Effects of TNNC1 - SNP on performance traits

Table 22 represents possible effects of TNNC1 SNP on the traits analyzed in the F2-DUPI resource population. This SNP is located at bp 716th in the intron part of the sequence with the accession No. AY958072.

In detail, body weight and ADG of the animals in any stages was not affected by this SNP ($P > 0.05$). However, several body composition and meat quality traits were found to associate with this SNP. Interestingly, loin eye muscle area values were highly influenced ($P = 0.001$) by the change of heterozygous C/G to homozygous G/G genotype (52.0 vs. 50.2 cm2, respectively). Moreover, other traits in body composition including lean content, loin weight and estimated lean content of carcass that are related to eye muscle area were shown to be significantly associated with this SNP with P values of 0.009, 0.014 and 0.001, relatively. In the same trend with eye muscle area traits, the differences existed only between C/G and G/G genotype group but not with C/C animals. However, fat area and fat: meat ratio were similar among animals with different genotypes ($P > 0.05$). For meat quality, the results showed that this SNP had no effects on meat colour (OPTO value), shear force, drip loss and cooking loss. In LD

muscle, pigs carrying C/C genotypes appeared to have high pH value, but the difference did not reach a significant level ($P = 0.066$). Also in this muscle, the conductivity values at both measuring time 45 min and 24 h p.m. were significantly associated with the changes of genotypes ($P = 0.017$ and 0.006, respectively). This trait, in addition, was lower ($P = 0.003$) in *Semimembranosus* muscle 24 h p.m. in animals having C/C genotypes compared with C/G and G/G animals (3.5 vs. 5.1 and 4.9, respectively).

Table 22: Effects of TNNC1 genotype on growth, body composition and meat quality traits (least squares means ± standard error)

Trait	Genotype			*P*-Value
	C/C	C/G	G/G	
Body weight (kg)				
Birth	1.62 ± 0.09	1.51 ± 0.07	1.50 ± 0.07	0.163
Weaning	8.67 ± 0.53	8.84 ± 0.38	8.85 ± 0.37	0.913
Test start	25.5 ± 1.3	26.1 ± 1.0	26.1 ± 1.0	0.807
Slaughter	111.1 ± 0.6	111.0 ± 0.3	111.0 ± 0.3	0.636
Average daily gain, ADG (g/day)	596 ± 12	602 ± 8	601 ± 7	0.819
Body composition				
Carcass length (cm)	98.2 ± 0.5	97.6 ± 0.3	98.0 ± 0.3	0.247
Eye muscle area (cm^2)	51.7 ± 1.1^{ab}	52.0 ± 0.8^{a}	50.2 ± 0.8^{b}	0.001
Fat area (cm^2)	15.6 ± 0.7	16.0 ± 0.5	15.5 ± 0.5	0.195
Fat : meat ratio	0.31 ± 0.02	0.31 ± 0.01	0.31 ± 0.01	0.865
Auto FOM lean content	65.0 ± 1.0^{ab}	64.5 ± 0.6^{a}	63.0 ± 0.5^{b}	0.009
Loin eye area (kg)	6.33 ± 0.10^{ab}	6.30 ± 0.05^{a}	6.16 ± 0.05^{b}	0.014
Meat quality				
MC_{Opto} (%)	70.1 ± 1.4	69.5 ± 0.8	68.9 ± 0.7	0.546
Shear force (N)	33.4 ± 1.8	35.6 ± 1.3	34.8 ± 1.3	0.276
Drip loss (%)	2.0 ± 0.2	2.2 ± 0.2	2.1 ± 0.1	0.506
Cooking loss (%)	24.5 ± 0.6	24.7 ± 0.5	24.9 ± 0.4	0.606
$pH1_{LD}$	6.63 ± 0.05	6.53 ± 0.04	6.54 ± 0.04	0.066
$pH24_{LD}$	5.52 ± 0.02	5.50 ± 0.01	5.49 ± 0.01	0.509
$pH24_{S}$	5.62 ± 0.03	5.61 ± 0.02	5.62 ± 0.02	0.935
$C1_{LD}$ (mS/cm)	4.1 ± 0.2^{b}	4.5 ± 0.1^{a}	4.3 ± 0.1^{ab}	0.017
$C24_{LD}$ (mS/cm)	2.6 ± 0.2^{b}	3.1 ± 0.2^{a}	2.9 ± 0.2^{ab}	0.006
$C24_{S}$ (mS/cm)	3.5 ± 0.6^{b}	5.1 ± 0.4^{a}	4.9 ± 0.4^{a}	0.003

[a, b] Means in the same row with different supersripts differ, $P < 0.05$

4.4.4 Association analysis of CKM

4.4.4.1 Allele and genotype frequency of the CKM polymorphic site

Allele and genotype frequency of CKM – SNP is displayed in Table 23. The distribution of two alleles "A" and "G" was 44 and 56%, respectively. However, there was a large range in genotype frequency from 7% (A/A) to 74% (A/G). Low frequency of homozygous animals G/G was also recognized (19%).

Table 23: Allele and genotype frequency of CKM gene in the F2-DUPI resource population

No of pigs	Allele frequency		Genotype frequency		
	A	G	A/A	A/G	G/G
329	0.44	0.56	0.07	0.74	0.19

4.4.4.2 Effects of CKM - SNP on performance traits

The effects of the CKM genotype on different phenotypes are shown in Table 24. In general, this SNP was more related to body composition, especially fatness traits rather than daily gain or meat quality traits. Results support the idea that backfat deposition accelerates heavily in the later growth stage. Pigs carrying A/A genotype offered greater slaughter weight and further higher fatness traits including backfat thickness, fat depth at neck region, at rib 13/14 and above loin area compared to those having A/G or G/G genotype ($P < 0.05$). Indeed, animals containing genotype A/A had significantly larger fat area ($P = 0.013$) than the other two genotypes A/G and G/G (17.3, 15.8 and 15.5, respectively). The same tendency of difference among these genotypes was also observed for fat: meat ratio ($P = 0.008$) and fat thickness above loin eye area ($P = 0.037$). In addition, higher values for average backfat and backfat depth at neck region in homozygous A/A animals were found, but the difference did not reach a statistical level ($P > 0.05$). Fat depth at rib 13/14 of heterozygous pigs was similar to those produced by homozygous pigs, which were different from each other in such a way that A/A animals had deeper fat than G/G animals (2.86 vs. 2.52). In contrast, animals

carrying the A/A genotype showed lower estimated lean meat content and dressing percentage than those with A/G and G/G genotypes ($P < 0.01$). Other meat quality traits such as meat color, shear force and pH were not affected by any genotype.

Table 24: Effects of CKM genotype on growth, body composition and meat quality traits (least squares means ± standard error)

Trait	Genotype			*P*-Value
	A/A	A/G	G/G	
Body weight (kg)				
Birth	1.57 ± 0.09	1.50 ± 0.06	1.56 ± 0.07	0.417
Weaning	9.08 ± 0.56	8.86 ± 0.40	8.90 ± 0.42	0.814
Test start	26.9 ± 1.3	25.7 ± 0.9	26.4 ± 1.0	0.510
Slaughter	112.4 ± 0.5[a]	110.8 ± 0.3[b]	110.8 ± 0.4[b]	0.007
Age at test start (day)	71.7 ± 1.2	71.7 ± 1.0	72.6 ± 1.0	0.351
Age at slaughter (day)	182.8 ± 3.7	181.7 ± 2.5	184.8 ± 2.7	0.518
Average daily gain, ADG (g/day)				
ADG 1	247 ± 17	252 ± 12	244 ± 13	0.751
ADG 2	357 ± 18	342 ± 12	340 ± 13	0.574
ADG 3	777 ± 21	781 ± 15	764 ± 16	0.534
ADG 4	609 ± 12	605 ± 8	597 ± 9	0.566
FCR from 30 to 105 kg	2.68 ± 0.06	2.67 ± 0.04	2.67 ± 0.04	0.986
Body composition				
Carcass length (cm)	97.8 ± 0.6	98.0 ± 0.3	97.7 ± 0.4	0.826
Eye muscle area (cm^2)	49.4 ± 1.2	51.3 ± 0.8	51.1 ± 0.9	0.187
Fat area (cm^2)	17.3 ± 0.7[a]	15.8 ± 0.5[b]	15.5 ± 0.5[b]	0.013
Fat : meat ratio	0.35 ± 0.02[a]	0.31 ± 0.01[b]	0.31 ± 0.01[b]	0.008
Average backfat thickness (mm)	2.22 ± 0.07	2.10 ± 0.05	2.10 ± 0.05	0.073
Backfat depth at neck region (mm)	3.58 ± 0.11	3.38 ± 0.07	3.34 ± 0.07	0.070
Fat depth at rib 13/14 (mm)	2.86 ± 0.15[a]	2.68 ± 0.10[ab]	2.52 ± 0.11[b]	0.050
Fat thickness above loin area (mm)	1.23 ± 0.07[a]	1.09 ± 0.05[b]	1.09 ± 0.05[b]	0.037
Dressing percentage (%)	75.8 ± 0.4[b]	76.9 ± 0.2[a]	76.9 ± 0.2[a]	0.008
Estimated lean content of carcass (%)	57.6 ± 0.6[b]	59.0 ± 0.4[a]	59.2 ± 0.5[a]	0.009
Meat quality				
MC_{Opto} (%)	70.9 ± 1.4	69.7 ± 0.8	68.2 ± 0.9	0.170
Shear force (N)	34.0 ± 2.0	34.2 ± 1.4	36.3 ± 1.5	0.297
$pH24_S$	5.64 ± 0.04	5.63 ± 0.02	5.60 ± 0.02	0.472

[a, b] Means in the same row with different supersripts differ, $P < 0.05$

4.5 Linkage mapping of TNNC1

Data obtained from genotyping of F1 and F2-DUPI resource population was used for linkage mapping of TNNC1 gene. This gene has been mapped in human chromosome 3. By using human and pig comparative map (http://www.toulouse.inra.fr/lgc/pig/compare/compare.htm), the TNNC1 gene was hypothsized to be on SSC13. A two-point linkage analysis, sex averaged, was carried out using CRI-MAP package (Version 2.4). The result showed that this gene is close to marker SW344 with the recombination fraction of 0.16 and LOD score of 9.74. Thus, by linkage mapping, the TNNC1 gene was assigned to SSC13 (Figure 19).

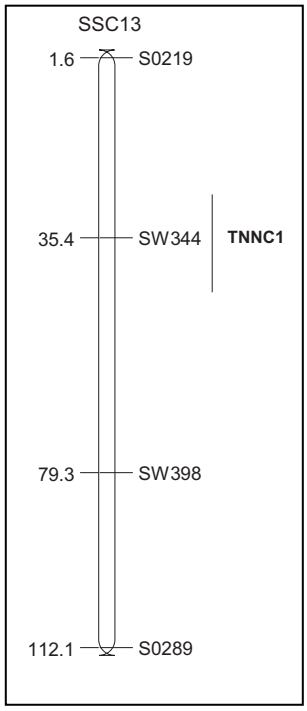


Figure 19: Linkage mapping of TNNC1 on SSC13

5 Discussion

5.1 Transcript abundance of MyHC isoforms

The growth and weight of muscle is mainly determined by the number and size of the muscle fibers and prolificacy of satellite cells. The proportion of different muscle fibers, their structure, and functional properties affect the animals' growth performance and are endogenous factors on post mortal meat quality traits (Lengerken et al. 1994). The dramatic improvement of growth performance and lean content in pig breeding is suspected to coincidence with altered meat quality. Inconsistency of results regarding the relationship of muscle fiber traits and meat quality and muscularity demonstrates the difficulty to determine the most advantageous muscle fiber type. The fact that some muscle fibers cannot be assigned to a particular type due to intermediate properties and the recent findings of four isotypes of adult MyHC protein in porcine muscle while conventional muscle fiber typing just classifies three types of fibers, demonstrates the needs to define new phenotypes in order to understand the relationship of muscle structural and functional properties, meat quality and quantity and the genetic control.

5.1.1 Muscle fiber composition in different breeds

The breed covers probably the main proportion of genetic factors affecting the muscle fiber composition of a certain muscle. Pietrain is well known for its muscularity and leanness while Duroc is favourable in meat quality in terms of marbling, tenderness and juiciness. The third breed, Mongcai, is a popular local breed in the Central coastline, the Red River delta and other Northern provinces of Vietnam with favored characteristics of high prolificacy, good adaptation to poor-quality feed and disease resistance but low performance with small body size and low growth rate. Genetically, Pietrain, Duroc and Mongcai are ostensibly distinct breeds in growth and muscularity, enormous genetic variation of each breed could account for the variation of MyHC relative expression across breeds.

The relative number of white fibers (IIx and IIb types) was indicated to contribute up to 85.2% in Pietrain (Müller et al. 2002) while only 8.5% of type I was identified.

Moreover, in Duroc muscle, the composition of muscle fiber types was slightly different (i.e.14.5% for type I and 81.5% for type IIx and IIb) from that of Pietrain (Chang et al. 2003). Results from the present study are comparable (86.3 and 77.8% for IIb + IIx in Pietrain and Duroc, respectively) to findings that in modern pig breeds, fiber composition has directed to a higher proportion of type IIb fibers (Ruusunen and Puolanne 2004). Similar profiles of muscle fiber types were reported in other commercial purebreeds including in Large White (Chang et al. 2003, Lefaucheur et al. 2002, Lefaucheur et al. 2004), Landrace and Yorkshire (Ruusunen and Puolanne 1997). In addition to the identification of muscle fiber composition in commercial pigs, the proportion of these fibers was also detected for the native Mongcai breed, in which, a dramatic change in relative expression profile was found. Indeed, the proportion of IIx and IIb fibers in Mongcai pigs was lower (47.3%) compared to other conventional breeds as analyzed here and in other studies such as Berkshire (85.7%), Hampshire (75.3%) and wild boar (84.2%) (Chang et al. 2003, Müller et al. 2002, Ruusunen and Puolanne 1997). When taking only IIb fibers into consideration, the proportion in Meishan LD muscle (Lefaucheur et al. 2004) was almost in line with the findings in Mongcai in this study (17.1 and 11.4%, respectively); but in Meishan, type IIx fibers were shown to be the most prominent (61.1%) (Lefaucheur et al. 2004) while in Mongcai type I, IIa and IIx fibers are more frequent.

The percentage of MyHC IIb is an important feature because it contributes to increase of muscle mass, which is desired by animal breeders. Kristensen et al. (2002) described that large muscle fibers and a high growth rate are also associated with higher protein turnover which may increase the synthesis of proteolytic enzymes and thereby have a positive effect on meat tenderness. Nevertheless, this conclusion is not always in agreement with other experiments. For instance, Chang et al. (2003) concluded that color characteristics, better water holding capacity and better tenderness were positively related to the presence of oxidative fibers and hence the main favorable fiber types were IIa and IIx. The sensory meat quality is closely related to the content of intramuscular fat, but various studies showed different outcomes regarding the location of lipids in fiber types. While Henckel et al. (1997) reported the frequency of IIb fiber and intramuscular fat content were positively correlated, Essén-Gustavsson et al. (1994) found lipids present mainly in type I/slow and some IIa fibers. Mongcai is known to have higher intramuscular fat and thereby show highly preferred meat quality but

unsatisfactory muscularity in comparison with commercial breeds with high muscularity but inferior meat quality. Although the lipid content was not determined in the current study, high percentage of IIa fiber from Mongcai muscle might suggest its important role in lipid storage. High values of intramuscular fat found in a cross of Duroc x Berlin Miniature Pigs additionally supported this view (Fiedler et al. 2003).

5.1.2 Muscle fiber composition of large and small muscles within breed

The muscle fiber type distribution in commercial crossbred pigs was almost similar with that of pure breeds. For example, Ryu et al. (2004) showed that the greatest percentage of myofibers was on white fiber IIx and IIb with a value of 80.2 % in a Duroc x (Yorkshire x Landrace) cross. A slightly lower proportion was previously reported by Fiedler et al. (1999) for a cross of Pietrain x Landrace (75%). Results from the current study indicated the trend that the contribution of IIx and IIb fiber in DUPI were lower compared to the pure breeds in either Duroc or Pietrain, and vice versa type I fibers were elevated up to 15% in the DUPI. However, differences were not statistically different at $P < 0.05$. In the DUMI resource population, there was evidence that the genetic background of Berlin Miniature Pigs led to decreased IIb fiber proportion (66.7%) whereas the relative expression of type I increased to 25.9%. Correspondingly, muscle fibers detected by histochemical differentiation revealed a contribution of 71% (type IIb and IIx) and 15.3% (type I). Furthermore, this study also revealed the fact that the difference between large and small eye muscle area was due to a different proportion of type IIb fiber. By analyzing well selected sib-pairs being specifically discordant in the trait eye muscle area but similar in various other traits that are possible factors in altering the fiber proportion, for the first time, it is demonstrated that IIb is the determinant fiber contributing to the differentiation of large and small eye muscle area in the pig.

In this study the muscle fiber composition of the same muscle (LD) between two pig groups within two populations (DUPI or DUMI) was examined. There are potentially several factors affecting the fiber proportion including birth weight, growth rate and slaughter weight. Although birth weight was indicated to have an association with enlarged muscle fiber area (Gondret et al. 2006), its influence on muscle fiber

composition of LD muscle and other muscles such as *Longissimus lumborum or Rhomboideus* was not found (Bee 2004). Similarly, the growth rate also has a given effect on the cross-sectional area of fibers especially on IIa, which was shown to increase with the increase of growth rate (Ruusunen and Puolanne 2004). But the IIa fibers are minor with low number shown in most studies, so they might not have strong effects on muscle size. Finally, in one study done by Ryu et al. (2004), they discovered that muscle fiber composition was not significantly associated with carcass weight and loin eye muscle area. Increasing weight and age at slaughter significantly affected the CSA but did not change the numerical percentage of any fibers (Čandek-Potokar et al. 1999). Thus, combining available findings with the controlled pig selection in this study, these affecting factors can be excluded in altering the muscle fiber proportions.

5.1.3 Muscle fiber typing by mATPase staining and the consistency between real-time RT-PCR and histochemistry methods

Various methods of muscle fiber differentiation do actually describe different phenotypes of the muscle. Since muscle fiber types are characterized by the content of different MyHC isoforms, their equipment with different enzymes, structural proteins and organelles that warrant specific biochemical and biophysical properties of the fibers, a combination of different methods to examine the fiber type proportions might give the most comprehensive characterization of muscle mass and further analysis of the genetic control. Conventionally, histochemistry by mATPase stability is used in most studies since many years and porcine fibers could be classified into type I, IIa and IIb (Fazarinc et al. 1995, Lefaucheur et al. 1991). For a more accurate muscle typing to distinguish pure IIx and IIb, and the hybrid of these two fibers, immunocytochemistry with specific antibodies and quantitative real-time PCR has been applied more recently for porcine fiber classification (Lefaucheur et al. 2004, Toniolo et al. 2004). The fiber proportion in the present research was almost in agreement with findings reported by Chang et al. (2003) and Lefaucheur et al. (2004) regardless of slight variation likely to be due to breed-specific effects.

The histological and quantitative real time assays were compared to estimate the proportion of fiber types in LD porcine skeletal muscle. The highly significant correlations of the corresponding MyHC isoforms evidenced the quite high consistency

of the two methods. The real time RT-PCR assay represents a new phenotype that is close to the effect of genes probably more suitable to unravel the genetic background in variation of traits related to muscle and meat properties depending on muscle fiber distribution. However, this assay does not provide information on the fiber size and fiber number.

5.2 Microarray experiment

Recently, construction and applications of porcine microarrays towards the muscle fiber type distribution traits and developmental stage (Bai et al. 2003, Cagnazzo et al. 2006) and differential expression in muscle depending on muscle in divergent breeds (Lin and Hsu 2005) have been reported. In an attempt to genetically detect genes that contribute differently to the performance of large and small muscle area in pigs, Ponsuksili et al. (2005) constructed a liver array (home-made array) with the assumption that genes active in liver potentially affect properties in relation to body composition and muscularity. Liver and muscle share a significant proportion of metabolic activities. Therefore, in the present study this array was used to examine the trait-associated expression profile of muscle to obtain genes related to porcine muscularity. Microarray hybridizations of labeled cDNAs from discordant sib-pairs with superior and inferior loin eye muscle area were performed with the aim to detect differentially expressed genes active in these kinds of muscles.

5.2.1 Validation of microarray results by real-time RT-PCR

Gene expression studied by microarray often results in a list of genes that appear to be differentially expressed. Among several available methods of assessing RNA expression such as Northern blot analysis or conventional RT-PCR, real-time RT-PCR is preferable because it is rapid and sensitive with minimal starting template (Abruzzo et al. 2005, Rajeevan et al. 2001). In the present study, real-time RT-PCR was applied to quantify the transcript abundance of differentially expressed genes not only in samples used in microarray but also in independent samples for stronger validation of the analyzed results. In total, three genes were quantified and out of these, two genes FTH1

and FHL1C were confirmed. The other gene, RAMP1 showed large variation of transcript abundance in large and small eye muscle area leading to a non-significant difference between the two groups. This is not surprising because in the microarray experiment probes were pooled, the variation in gene expression was hidden. Therefore, in order to have confirmed results of up and down regulated genes in a microarray study, an appropriate and independent method should be taken into account at individual expression level (Ponsuksili et al. 2005).

5.2.2 Expression of FTH1

Ferritin heavy chain is a protein that is expressed in many tissues and plays an important role in iron storage and metabolism. The two subunits of ferritin namely light and heavy chain can be regulated at both transcriptional and translational levels (White and Munro 1988). Due to its inherent ferroxidase activity participating in iron oxidation (Strube et al. 2002), ferritin heavy chain is therefore necessary in mobilization of iron to and from the ferritin protein complex.

Little documentation about the expression of this gene in animals, especially in relation with muscular characteristics, has been published. A study by Kitahara et al. (1995) showed high expression of FTH1 in rat skeletal muscle after denervation, a factor resulting in atrophy of muscle fibers and other contractile changes. In pigs, FTH1 was identified to be differentially expressed in the anterior pituitary of sows, in which the level of mRNA is reduced as a result of selection for increased ovulation rate and embryo survival (Bertani et al. 2004). Functionally, the expression of FTH1 appears to be associated with the characteristics of muscle fiber types, particularly with the presence of mitochondria in each fiber. The importance of iron in mitochondrial function is well known by a mechanism that mitochondria mobilizes iron from ferritin, and iron is used for the synthesis of heme and the generation of iron-sulfur, which are important for the optimal activity of electron transfer complexes (Lill and Kispal 2000, Ponka 1997). Indeed, both iron deficiency and iron excess can lead to damaging mitochodira and mitochondrial DNA in rats (Walter et al. 2002). Type I and IIa fibers contain more mitochondria than type IIx / IIb fibers and thus need more iron. In the present study, low performance pig (small eye muscle area) showed higher relative

expression of type I fibers than in high performance pigs. The established relationship between iron content and mitochondria can contribute to explain the result that FTH1 is up-regulated in pigs having small muscle area.

5.2.3 Expression of FHL1C

Both FHL1 (SLIM1) and FHL1C, a new variant of FHL1, are expressed in skeletal muscle (Krempler et al. 2000). The FHL proteins consist of four repeats of a LIM domain and an N-terminal single zinc finger that meets the consensus of the C-terminal half of the LIM domain motif and hence is designated as Four-and-a-Half-LIM proteins (Morgan and Madgwick 1999b). So far, four different isoforms of FHLs have been described in various muscles. For instance, FHL1 and FHL3 are two main isoforms in skeletal muscle (Lee et al. 1998, Morgan and Madgwick 1999b), FHL2 is mainly expressed in cardiac muscle (Chan et al. 1998) and FHL4 exclusive expression is found in testis (Morgan and Madgwick 1999a).

At early stage of development, the FHL1 gene was reported to be higher transcribed in Duroc muscle at birth and at postnatal day 7 than in Taoyuan (Lin and Hsu 2005). This is supported by the existence of a relationship between increased FHL1 transcription and skeletal muscle growth. McGrath et al. (2003) suggested the involved function of this gene in the $\alpha_5\beta_1$-integrin-mediated signaling pathways and in playing a key role in the early stages of skeletal muscle differentiation. An elevation of FHL1 mRNA expression was found to be associated with postnatal muscle growth (Morgan et al. 1995). This was later supported by Loughna et al. (2000), who demonstrated functional regulation of FHL1 in skeletal muscle. Recently, its function was identified as a novel regulator that binds myosin-binding protein C and regulates myosin filament formation and sarcomere assembly (McGrath et al. 2006). In rats, higher transcripts in type I and IIa fibers of the m. soleus compared to IIb fiber of the m. gastrocnemius may indicate that this gene is highly expressed in adult oxidative rather than glycolytic muscle fibers (Loughna et al. 2000). Similarly, Campbell et al. (2001) and Schneider et al. (1999), by observing the expression of FHL1 in mice, concluded that mRNA for LIM protein in mixed red muscle were many folds higher that that of white quadriceps muscle. In a knockout model of muscle LIM protein in mice, CSA of type I and IIa fibers was found

to be small in knockout muscle but there was no effect of gene knockout on IIx and IIb fibers (Barash et al. 2005). Furthermore, muscle LIM protein was indicated up-regulated during transition from fast to slow fibers, suggesting its possible function involved in the maintenance of slow fibers (Schneider et al. 1999, Willmann et al. 2001). In the current experiment, results of muscle isoform quantification by real-time PCR together with microarray analysis strongly supported the findings that FHL1 is highly expressed in slow fiber muscle. Type I fiber transcripts were significantly higher in small muscle group whereas the transcripts of IIb fiber were more abundant in large muscle animals. Thus, based on the convincing evidence of differential expression, further investigation of this gene in association with muscle traits is recommended.

5.3 Relative expression of TNNC1 and CKM in Pietrain and Mongcai

The European breed Pietrain is well known for its high muscularity while the Mongcai is recognized as a fatness breed in Vietnam. These two breeds display remarkable differences in postnatal muscle growth; therefore expression profile of differentially expressed genes may be used as candidates for muscle growth and meat quality traits.

5.3.1 Expression of TNNC1

Troponin C is highly expressed in cardiac and skeletal muscle. Both cardiac and skeletal muscles are regulated by changes in the intracellular concentration of Ca^{2+} in the fibers. Troponin, the central regulatory protein of striated muscle contraction, is a component of thin filaments together with actin and tropomyosin (Farah and Reinach 1995). Vertebrate troponin contains three subunits TnI (troponin I), TnT (troponin T) and TnC (troponin C) (Hooper and Thuma 2005), in which when calcium is bound to specific sites on TnC, the binding complex will become a molecular switch that regulates muscle contraction and produces force and movement. The fast (TnC-f) and slow/cardiac (TnC-s) isoforms are the only two isoforms existing in TnC. Differences in the number of active calcium binding sites (Moss et al. 1995) and the affinity for TnI (Tobacman 2002) of these two isoforms result in different properties of the contractile

apparatus including sensitivity to calcium (Moss et al. 1986) and contraction (Metzger 1996).

In a study of screening genes differentially expressed in swine tissues between the muscle and fat of early growth stage, Kim et al. (2005) found that genes needed as energy source in the formation of growth, including TNNC1 are expressed mainly in fat tissues. These authors further suggested these genes as a temporary bridge contributing to the formation of muscle growth and thereby the relation between muscle and fat growth is symbiotic. In addition, the expression of TNNC1 appeared to have a link with different MyHC isoforms. Furukawa and Peter (1971) stated that troponin activity in skeletal muscle of guinea pig is related to three histochemical fibers in such a way that intermediate and red fibers have lower troponin activities than white fibers. Moreover, in a protein expression study in rat skeletal muscle, O'Connell et al. (2004) concluded a close relationship between MyHC and TnC isoform composition. They found that only TnC-s is expressed in fibers containing exclusively slow MyHC isoform. In the present study, the expression of TnC-s at mRNA level was analyzed in two extreme muscles, which were significantly different in the proportion of MyHC. LD muscle from Mongcai pigs contained higher percentage of slow fiber (type I) and conversely lower proportion of white fibers (type IIb) compared to those from Pietrain. Supposed that the translational efficiency from mRNA to protein were equal in all MyHC isoforms, higher expression of TNNC1 in Mongcai would therefore be expected. However, the findings were in a reverse direction and thereby were not in agreement with the conclusion of O'Connell et al. (2004). In this study, the expression of MyHC isoforms might be mismatched with TnC isoforms similarly to published data by Danielibetto et al. (1990) and Geiger et al. (1999).

5.3.2 Expression of CKM

Creatine kinase (CK) isoenzymes are present in all vertebrates. Creatine is mainly synthesized in liver and kidney but is not produced by muscle. It is transported and taken up by tissues via an active system known as creatine transporter (Fitch et al. 1968), and plays a role in maintaining intracellular ATP levels, especially in tissues with high energy demand, or functions as a temporal energy buffer and a energy

transport system (Wallimann et al. 1992). Depending on the energy needs of the tissue, one of these two functions can act interchangeably. For example, the buffer function may dominate in glycolytic muscle but reduce activity in oxidative fibers in comparison with the transport function (Wallimann et al. 1992).

The concentration of total creatine (phosphocreatine + creatine) is different among mammalian skeletal muscle and seems to parallel with muscle glycolytic activity, thus the expression of creatine could be responsible for the variations in total creatine in the muscle fiber types (Brault and Terjung 2003, Ventura-Clapier et al. 1998, Wyss and Kaddurah-Daouk 2000). In human muscle trained for long distance running, a significant decrease in total CK was recognized together with the conversion of fast-twitch to slow-twitch fibers (Apple and Rogers 1986). Furthermore, creatine supplementation increased the diameter of type II fibers but had no effect on type I fibers (Wyss and Kaddurah-Daouk 2000). An increase of mitochrondrial CK was also reported in chronically stimulated fast-twitch rabbit muscle (Schmitt and Pette 1985).

One of the classification schemes of skeletal muscle fibers is characterized by ATPase activity and contractile speed. In fact, high shortening speed of fast-twitch fibers is produced by high myosin ATPase activity and associated with muscle CK to supply ATP for contraction (Yamashita and Yoshioka 1991). In rats, Yamashita and Yoshioka (1991) demonstrated that total CK and muscle CK activities were highest in fast-twitch glycolytic fiber and conversely lowest in slow-twitch oxidative fibers. The present study supports this finding as higher expression of CK was found in pigs having greater proportion of type IIb fibers. The results are also in agreement with the fact that the content of CK and phosphocreatine (PCr) is high in glycolytic but relatively low in oxidative fibers (Ventura-Clapier et al. 1998) and that the role of CK as an energy buffer is greatest in muscles expressing the IIb isoform (Watchko et al. 1996). In an attempt to distinguish mammalian skeletal muscle fibers by the content of phosphocreatine, Kushmerick et al. (1992) proposed a 2-fold difference in the phosphocreatine content between muscles having high volume of type I and IIx fibers in contrast with those from IIa and IIb fibers. However, by this method type IIa and IIb, which differ in their metabolic activities, were indistinguishable making it vague to set up a relationship between CK and muscle fibers according to the classifying scheme discussed in the literature review.

5.4 SNP detection, genotyping and association analysis

SNP, comprising 80% of all known polymorphisms, contribute greatly to genetic variation and their density is estimated to be on average 1 every 1,000 base pairs in the human genome (Editorial 1998). Detection of SNP can be defined as a process of scanning for new polymorphisms and determination of the allele(s) of a known polymorphism in target sequences (Kwok and Chen 2003). A comparison of a part of the sequence among breeds within a species, known as comparative sequencing, is often used to detect SNP. When the position(s) as well as number of SNPs are available, selection of method for genotyping is a critical point to be taken into account since this step is likely a major part in any association study (Kwok 2001). In the current study, PCR-RFLP (Restriction Fragment Length Polymorphism) was applied successfully in genotyping three genes FTH1, TNNC1 and CKM.

5.4.1 Association analysis of FTH1

The two SNPs found in FTH1 gene are located in the 3' UTR region. These polymorphic sites were previously described (Ponsuksili et al. 2002, Rattink et al. 2001a, Rattink et al. 2001b). From the two detected SNPs in the F2-DUPI resource population, a construction of haplotype by using Merlin software (http://www.sph.umich.edu/csg/ abecasis/Merlin/tour/haplotyping.html) was performed resulting in 5 different haplotypes. These haplotypes together with two SNPs were then used for the association study.

The FTH1 gene was previously mapped to SSC2 (Ponsuksili et al. 2002) between the loci SW2443 (proximal) and SW240 (distal) with distances of 42.8 and 20.2 cM, where several QTLs related to ADG were reported (CasasCarrillo et al. 1997, Lee et al. 2003, Malek et al. 2001). Particularly, Pietrain QTL alleles were associated with muscle, high daily gain and low fat deposition (Lee et al. 2003). However, in the F2-DUPI population, evidence for a suggestive QTL for ADG was not detected in SSC2 (Liu, 2005). The effects of QTL on microstructural and biophysical muscle properties were additionally reported on SSC2. In a Japanese Wild Boar x LW intercross, Nii et al. (2005) provided an evidence (< 1% genome-wide scan) that the proportion of type IIa

fibers is affected by QTL (position 59.1 cM) on SSC2. These authors also reported on QTL for the number of type IIb fibers (position 103.5 cM) and relative area of type IIa fibers (61.1 cM). Similarly, in a DUMI resource population, Wimmers et al. (2006) found QTL for muscularity, i.e. lean meat content and loin eye area at position 20 cM for both QTL on SSC2. These QTL results are interesting because in this study, the expression of FTH1 is different in muscle with different fiber type compositions. Its association with growth rate, shear force, meat color and pH together with these suggested QTLs help to propose FTH1 as a functional and probably a positional candidate gene for muscle growth and meat quality traits.

The level of FTH1 expression may affect iron storage and thereby animal performance. Iron is essential for the growth and development of most organisms and is present within the cells either in a form of complex iron-containing proteins and enzymes or in iron-storage proteins (Carrondo 2003). These proteins are crucial for biological functions including electron transfer reactions, gene regulation, oxygen metabolism and regulation of cell growth and differentiation (Beard 2001, Ponka 1999). A sufficient supply of iron is necessary and iron deficiency will result not only in reduced food intake but also in reduced feed efficiency (Beard et al. 1995, Rosales et al. 1999, Strube et al. 2002).

Because the evaluation of tenderness by a taste panel is costly and time-consuming, and owing to a rather high phenotypic correlation between shear force and tenderness, Warner-Bratzler shear force (WBSF) is often used as an effective index trait instead of the use of a panel (Boccard et al. 1981, Hovenier et al. 1993). Tenderness and mechanical properties of meat are principally influenced by connective tissue, myofibrils and their interaction (Sacks et al. 1988). One of the main components of muscle connective tissue that has been considered to contribute to the “back ground” of meat toughness is collagen (Dransfield et al. 1984, Torrescano et al. 2003). Collagen and different fibers appear during foetal development. During the last third of the gestation, in muscles of double-muscle animals, the number of bigger cells is higher leading to less extracellular space and thereby reduced quantity of collagen while slow twitch fibers, which are smaller, are surrounded by more collagen (Listrat et al. 1999). Although collagen represents only 2% of total protein in muscle, it is accountable for several textural changes in meat during heating (Powell et al. 2000). Therefore, the content of muscle collagen is important from the point of view of meat quality. The

important role of iron in collagen synthesis can be explained by the fact that (1) iron is required as cofactor for prolyl-hydroxylase and increased activity of the enzyme has been shown in various models of iron overload and (2) iron may promote gene transcription of mRNA expression for the formation of the procollagen chains (Weintraub et al. 1988).

A number of inconsistent outputs on the relationship between muscle fiber type composition and meat tenderness exist. The frequency of type IIb fibers has been shown to be negatively correlated with toughness in pigs (Karlsson et al. 1993) and in cattle (O'Halloran et al. 1997). Additionally, Maltin et al. (1998) and Strydom et al. (2000) reported a positive relationship between the frequency of percentage area of type I fibers and sensory tenderness and a negative relationship between the frequency of type IIb fibers and tenderness. Calkins et al. (1981) and Seideman et al. (1986) earlier found a negative correlation between frequency of IIb fibers and meat tenderness in various bovine muscles. More specifically, it was shown that a lower percentage of IIb fibers is in associated with increased shear force (Muir et al. 2000). In limb skeletal muscle of adult rats, collagen was found to have a greater amount in slow than in fast twitch muscles (Kovanen et al. 1980). These authors later observed the same difference on the level of individual fast twitch and slow twitch fibers within a single muscle (Kovanen et al. 1984). In brief, the association FTH1 and shear force can be summarized by a simple flow that ferritin is important for iron storage, which is in turn needed for collagen formation. Meat tenderness or shear force is affected by the collagen content and also has certain association with the proportion of muscle fiber types.

In addition, a negative relationship between collagen and pH_u probably causes a significant correlation between collagen and meat color in sheep carcass because low pH is accompanied by increased light reflectance and also enhanced conversion of myoglobin to metmyoglobin (Livingston and Brown 1981, Owen and Lawrie 1975). Meat color is the combination of many factors including the concentration of heme fragments, the chemical state of these pigments and physical properties of meat structure. The stage of pigments is affected by meat pH, and the reaction of the heme with other reactants like O_2 can influence the stage of heme iron (Fernandez-Lopez et al. 2004), which partly depends on FTH1 expression.

5.4.2 Detection of SNP in FHL1C

In order to screen for SNP of the FHL1C gene, the F2-DUPI, F2-DUMI resource population together with pure breeds of German Landrace, Duroc, Pietrain, Mongcai and wild boar were used for comparative sequencing. More than 95% out of 2555 bp of exon regions in the gene with accession number NM_214375 was sequenced. The rest of the nucleotides could not be sequenced because of the limitations in primer design to obtain an amplified product. However, the result was unexpected as no SNP was found in this region. Furthermore, with suspect that the mutation might occur in the promoter region, an amplification of 610 bp from the genomic DNA of DUPI, Pietrain, German Landrace and wild boar was sequenced, but still no SNP could be detected. Therefore, this gene seems to be conserved across breeds and does not follow the estimation of nucleotide diversity index on porcine SNP, which is about 1/609 bp (Fahrenkrug et al. 2002).

Recently, in a study of tumor cells at protein level, Shen et al. (2006) proposed a pathway, in which Scr tyrosine kinase (Scr) phosphorylates Crk-associated substrate (Cas) leading to decreasing expression of FHL1. With the hypothesis that one of these two factors could be responsible for the differential expression of FTH1 in the microarray and real-time RT-PCR in the current research, the Scr gene was selected and quantified. However, the difference of the Scr expression in two extreme groups of pigs with small and large eye muscle area was not significant (data not shown) and thus the involvement of this gene can be questioned. Further studies on the effects of Cas gene in controlling FHL1 expression is undertaken.

5.4.3 Association analysis of TNNC1

The documentations on the SNP as well as association of TNNC1 with performance traits in pigs are limited. Instead, most of researchers discussed about the role of this gene in clinical setting such as heart failure (Adamcova et al. 2006). In the present study, because the detected SNP of TNNC1 is located in the intronic region, it could not be the causative polymorphism. The association of such SNP with observed traits may be explained by the influence of intron on mRNA metabolism including initial

transcription, editing and polydenylation of the pre-mRNA, translation and decay of the mRNA product (Le Hir et al. 2003). Moreover, there are an increasing number of reports for the role of introns in regulating the expression level of a gene or tissue-specific expression pattern (Glazier et al. 2002, Greenwood and Kelsoe 2003, Pagani and Baralle 2004, Van Laere et al. 2003). Further research in farm animal additionally supported this view, i.e. Van Laere et al. (2003) evidenced a QTL affecting muscle growth, fat deposition and heart size in pigs is due to a SNP in the 3rd intron of the insulin-like growth factor-2 (IGF2) gene.

The SNP found in the intron of TNNC1 is associated with traits related to loin eye muscle area, which is of interest in porcine meat production. Studies on the association of different genes with this trait have been reported. For example, a SNP at nt 778 (A>C) transition of the thyroxine-binding globulin (TBG) gene was revealed to have an association with eye muscle area (Ponsuksili et al. 2005). Linkage and association analysis of the corticotropin-releasing hormone (CRH) gene showed its SNP in the QTL region that is highly significant with loin muscle area (Muráni et al. 2006). More specifically to the microstructural characteristics of muscle, Kłosowska et al. (2005) discovered a significant relation between the intronic SNP in the calpastatin (CAST) gene and the content of muscle fiber types in Stamboek pigs. In a half-sib analysis of the F2-DUMI resource population at 5% chromosome-wide level, Wimmers et al. (2006) found evidence for QTL on SSC13 influencing on microstructural traits related to muscle such as total number of fibers, diameter of fast-twitch glycolytic fibers and mean number of fibers per mm^2. Additionally, an earlier study done by Kurył et al. (2003b) suggested the effect of this locus on the proportion of fast-twitch glycolytic fibers in a bundle and hence may also affect the muscle properties.

Conductivity has been considered a useful parameter to estimate water holding capacity of meat. Charged compounds in meat fluids enhance electric current conductivity and thereby the higher the percentage of water in meat, the less resistant to a current the meat will be (Kusec et al. 2005). The SNP found in TNNC1 in the present research was associated with meat conductivity at both stage of 45 min and 24 h p.m. This is in accordance with QTL data by Yue et al. (2003a), who reported a significant relationship of QTL markers with conductivity close to the region where TNNC1 was mapped. Therefore TNNC1 can be proposed as a positional candidate gene for meat quality, particularly for conductivity.

The value of pH had a tendency to differ among genotypes although these figures were not statistically different. A QTL scan based on Duroc and Berlin Miniature pig on SSC13 showed evidence for QTL significant at the 5% chromosome-wide level with pH1 and pH24 in LD muscle (Wimmers et al. 2006). Practically, the suggestion that TnC isoforms may have an effect in conferring pH sensitivity of Ca^{2+} activated contraction in mammalian fast and slow muscle fibers has been evidenced by many authors (Metzger 1996, Palmer and Kentish 1994, Solaro et al. 1989). The possible mechanism on the alternation of TnC on pH sensitivity could be that the extent of acidic-pH-mediated reduction in Ca^{2+} binding to regulatory binding sites on TnC is independent of any isoforms (Metzger 1996).

Although TNNC1 was shown to associate with some economic traits, this effect may be due either to TNNC1 or to linked genes. In this study, the TNNC1 gene was mapped close to marker SW344 on SSC13, where the pituitary-specific transcription factor (PIT1) was mapped. The *RSa*I-PIT1 SNP was reported to have an association with loin muscle area (Yu et al. 1995). Moreover, the association of 14 traits of growth rate and carcass composition with PIT1 genotypes in a reference family of wild boar x Pietrain cross was presented by Brunsch et al. (2002) followed by the findings on the significant effect of PIT1 gene on daily gain, ham-covering fat, fat thickness over loin, meat content of carcass and ham (Pierzchała et al. 2003). However, based on the polygenic inheritance of quantitative traits (Yu et al. 1999) one of the hypotheses explaining for the PIT1 polymorphisms and economic traits could be its link with other genes that are involved in these traits (Franco et al. 2005). Further studies on the association of TNNC1 with economic traits with regard to the presence or absence of PIT1 polymorphisms are recommended.

5.4.4 Association analysis of CKM

The polymorphic site of CKM was detected in the promoter region. There is no information available on the SNP as well as association of this gene with performance traits in farm animals. In this study, CKM is highly expressed in Pietrain, a well known breed for muscularity compared to Mongcai, which produces a lot of fat. Pigs carrying genotype A/G and G/G had lower slaughter weight than those having A/A genotype. As

a consequence, meat percentage and other meat traits including estimated lean meat content and dressing percentage are higher in A/G and G/G pigs. Conversely, fatness trait values are higher in A/A pigs. These data reveal a systematical association between favorable (i.e. negative) effects on fatness and favorable (i.e. positive) effects on muscularity. Nevertheless, it should be mentioned that the frequency of A/A genotype is only 7% implying a low number of pigs with high fat content in the F2-DUPI resource population. In this case, the "A" allele contributes to a higher fat traits whereas the "G" allele is more involved in muscularity traits.

The results are also in agreement with those derived from QTL analyses. The CKM has been appointed as a skeletal muscle gene and has been mapped on SSC6 by somatic cell hybrid panel and radiation hybrid mapping (Davoli et al. 2002, Davoli et al. 2003). Around this region, there are QTLs for backfat traits (Óvilo et al. 2002, Yue et al. 2003b) and creatine phosphokinase, the only enzyme activity trait, is also mapped on SSC6 (Geldermann et al. 1996). These consistent results on the association of different genotypes with fatness and meat traits with QTLs confirm the potential role of CKM as a positional and functional candidate gene for these traits provided that this gene acts independently with other genes such as leptin receptor gene (LEPR), heart fatty acid-binding protein (H-FABP) and particularly the halothane (HAL) gene.

The fatness trait has been reported to associate with several genes in different porcine chromosomes. First, the significance of leptin (LEP) in SSC18 to meat: fat ratio and backfat was reported (Hardge et al. 1998, Jiang and Gibson 1999). In addition, a missense mutation in a region, which is highly conserved among the melanocortin receptor (MC4R) gene located in SSC1, results in significant difference in backfat depth of LW (Houston et al. 2004, Kim et al. 2000). In this study, though the CKM was associated with many fatness traits, its linked function with other genes previously known to affect on these traits were not covered. For example, in SSC6, LEPR and H-FABP are considered as candidate genes due to their position and association with fatness traits (Gerbens et al. 2000, Óvilo et al. 2002). The other locus on SSC6, RYR1 (ryanodine receptor 1), was assigned as a candidate gene for malignant hyperthermia syndrome (MHS), which is known as a porcine stress syndrome and as a cause of large losses in swine industry. This gene was indicated by several studies to have significant effects on backfat thickness (Dovc et al. 1996, Krenkova et al. 1999, Krenkova et al. 1998). When being homozygous (*nn*), RYR1 contributes to the increased water content

of lean muscle and suppresses fat deposition (Zhang et al. 1992) but it is more susceptible to poor meat quality, especially in terms of higher incidence of PSE in comparison with negative pigs (*NN*) (Sather et al. 1991a, Sather et al. 1991b). Likewise, the halothane carrier animals (*Nn*) had an advantage over negative pigs for feed efficiency, carcass yield and fat-free lean content but offered higher incidence of PSE (Leach et al. 1996, Pommier et al. 1998). Presently, in the F2-DUPI resource population data on the MHS test are not available, further studies on the possibly functional and positional link between CKM and RYR1 gene are encouraged.

5.5 Future prospects

Owing to their genetic relationships, muscle fiber characteristics, muscle growth and meat quality are preferable in practical breeding (Fiedler et al. 2004). However, body compostion and meat quality are currently two major problems for animal breeders when attemping to improve the production and performance. These traits are in part negatively correlated and can be measured only at post mortem, which make prior breeding selection impossible. Therefore, an intensive understanding on the microstructural properties of muscle fibers and their relationship with body composition and meat quality would be a further goal to achieve. In addition, it shoud be beared in mind that a high number of genes involved in the (negative) correlation between meat production and meat quality traits makes the identification of genes responsible for phenotypic variation and the investigation of basic molecular mechanisms a difficult thing. However, it is possible to include muscle fiber characteristics in breeding programs to improve meat quality and to preserve optimal production traits (Karlsson et al. 1999). Results from a simulated selection on the use of muscle fiber traits as additional selection criteria for muscle for muscle growth and meat quality (Fiedler et al. 2004) strongly supports this view and thereby stimulate more detailed studies on muscle fiber type especially on physiological mechanisms so that these characteristics can be effectively exploited in animal breeding programs. This study has demonstrated the ability of using muscle fiber as a new phenotype to unravel the genetic background on the variation of muscle-related traits. In the future, a combined analysis of QTL study with differential gene expression profiles derived from, i.e. microarray

experiments, can be used to predict muscle-related traits, especially meat quality in the live pigs, which in turn allows an early breeding selection.

6 Summary

The present work was carried out to study the expression of MyHC isoforms in the commercial and local pig breeds and in the same breed with extremely small/large loin eye muscle area. Furthermore, candidate genes derived from microarrays together with a functional candidate gene were analysed for SNPs and their association with performance traits studied.

For the identification of muscle fiber types, three pig breeds of Pietrain, Duroc and Mongcai and two crossbreeds of Duroc x Pietrain (DUPI) and Duroc x Berlin Miniature Pig (DUMI) were examined. Of the commercial breeds, Pietrain and Duroc, nine unrelated animals were sampled. Out of 598 F2-DUPI and 420 F2-DUMI, six discordant sib-pairs from each cross, representing extremes for the trait loin eye muscle area (large vs. small) were selected for MyHC quantification.

Animals of two commercial breeds and a native breed were examined to be different in traits related to muscularity and meat quality. In both commercial breeds Duroc and Pietrain, abundance of MyHC type IIb accounted for more than half of the MyHC transcripts (59.7 and 65.4%, respectively). In terms of slow fiber, DUMI had a significantly higher proportion of MyHC I isoform than other breeds, with the exception of Mongcai. The native pig, Mongcai, showed lowest MyHC IIb transcript abundance but highest proportion of other isoforms compared to the other breeds and crosses. Owing to genetic characteristics, a larger percentage of muscle area from DUPI than DUMI was observed. However, this did not affect the shift of direction of fiber type composition. The discordant sib-pairs in the DUPI and the DUMI population differed significantly ($P < 0.05$) in the relative abundance of MyHC type IIb transcripts. In the DUPI resource population, similar transcript level of MyHC I was found in both large and small muscles, but in DUMI these figures were statistically different. By ATPase staining after acidic preincubation (pH 4.6) three fiber types were distinguished. The correlations between these phenotypes ranged between 0.53 and 0.72 and were highly significant ($P < 0.05$) for the corresponding pairs of muscle fibers characterized by histochemistry and transcript abundances of the MyHC isoforms.

In the microarray experiment, four discordant sib-pairs of F2-DUPI animals were used. These animals were different in loin eye muscle area (large vs. small) but were similar in age, slaughter weight and carcass weight. Three genes (FTH1, FHL1C and RAMP1)

were shown to be highly expressed in the small muscle animal group and are related to cellular communication and regulation of metabolism. Two of them, FTH1 and FHL1C were further validated by real-time RT-PCR. Moreover, the TNNC1 gene was selected from its fold change difference (1.8) in a preliminary hybridization (QIAGEN array) between the commercial breed Pietrain and a local breed Mongcai. The expression levels of RNAs among the pigs were in a large range but followed the trend that TNNC1 is highly expressed ($P < 0.01$) in Pietrain compared to Mongcai. As a result, an average fold change value of 6.0 was generated and therefore TNNC1 was concluded as an up-regulated gene in Pietrain. In addition to selecting genes derived from microarray results as candidate genes, literature search was also done and the CKM was chosen based on its positional and proposed functional relationship to skeletal muscle in pigs. By real-time RT-PCR, the CKM was found to have low transcript abundances in Mongcai compared to Pietrain. The significant ratio ($P < 0.001$) in relative mRNA level of CKM between the two extreme pig breeds suggests its potential role in muscle and meat quality.

For all genes, the SNPs were detected in the F2-DUPI resource population. In detail, two SNPs were discovered in the 3'UTR of the FTH1 gene. The other gene derived from microarray, FHL1C did not show any SNP in all exons and some intron regions. For TNNC1, four SNPs were detected in the intron part. Out of these, the 716 SNP was selected for genotyping. Finally, one SNP was found in the promoter region of the CKM gene.

In the association study, the FTH1-SNP1 had no effect on any traits analyzed. However, among parameters for body weight, FTH1-SNP2 was associated with animal weight at the start of the experiment (72 days). Moreover, this SNP had an effect on the growing stage when piglets were weaned until they were allocated in the test box. The overall daily gain of animals bearing genotype G/G was significantly higher than those with genotype A/A (611 g vs. 589 g, respectively). For meat quality traits, shear force was statistically related to the genotypes, especially with homozygous pigs ($P = 0.025$).

When combining two SNPs to construct a haplotype, some traits were found to have a significant relation with a certain genotype. For example, the age of pigs at slaughter was significantly associated with haplotype GT/GT and AC/AC. Haplotype AC/AC and CG/GT also affected ADG3 and ADG4 but not the early stages of development. High daily gain, especially in the last period of growth made haplotype CG/GT the lowest

value in terms of FCR (2.52 kg) with the statistic level $P = 0.026$. In addition, meat color was found to be associated with haplotypes, in which the GT/GT haplotype offered higher value than the CG/GT haplotype (70.7 and 65.8, respectively). Different haplotypes were also in association with pH24 value in *Semimembranosus* muscle with lowest pH in animals with CG/GT haplotype (5.55) and highest pH value in the GT/GT haplotype (5.64).

For the TNNC1 SNP, loin eye muscle area values were highly influenced ($P = 0.001$) by the change of heterozygous C/G to homozygous G/G genotype (52.0 vs. 50.2 cm^2, respectively). Moreover, other traits in body composition including lean content, loin weight and estimated lean content of carcass that are related to eye muscle area were shown to be significantly associated with this SNP with P values of 0.009, 0.014 and 0.001, relatively. In the same trend with eye muscle area traits, the differences existed only between C/G and G/G genotype group but not with C/C animals. Also in this muscle, the conductivity values at both measuring times 45 min and 24 h p.m. were significantly associated with the changes of genotypes. This trait, in addition, was lower in *Semimembranosus* muscle 24 h p.m. in animals having C/C genotypes compared with C/G and G/G animals (3.5 vs. 5.1 and 4.9, respectively).

Generally, the CKM SNP was more related to body composition, especially fatness traits rather than daily gain or meat quality traits. Results support the idea that backfat deposition accelerates heavily in the later growth stage. Pigs carrying A/A genotype offered greater slaughter weight and further higher fatness traits compared to those having A/G or G/G genotype. Indeed, animals containing genotype A/A had significantly larger fat area than other two genotypes A/G and G/G (17.3, 15.8 and 15.5, respectively). The same tendency of difference among these genotypes was also observed for fat: meat ratio and fat thickness above loin eye area. Fat depth at rib 13/14 of heterozygous pigs was similar to those produced by homozygous pigs, which were different from each other in such a way that A/A animals had deeper fat than G/G animals (2.86 vs. 2.52). In contrast, animals carrying the A/A genotype showed lower estimated lean meat content and dressing percentage than those with A/G and G/G genotypes ($P < 0.01$).

In conclusion, by analyzing well selected sib-pairs being specifically discordant in the trait eye muscle area but similar in various other traits that are possible factors in altering the fiber proportion, we have shown for the first time that IIb is the determinant

fiber contributing to the differentiation of large and small eye muscle area in the pig. Moreover, the candidate gene approach, particularly the findings of differentially expressed genes by using microarray in two extreme muscle groups has resulted in indentification of genes that were associated with economically important traits in pig production.

Zusammenfassung

Diese Arbeit wurde durchgeführt, um die Expression von MyHC Isoformen in kommerziellen europäischen und lokalen vietnamesischen Schweinerassen sowie den gleichen Rassen mit extremen großen und kleinen Kotelettflächen zu untersuchen. Es wurden zusätzlich aus Mikroarrays abgeleitete Kandidatengene und funktionelle Kandidatengene auf SNPs und ihre Assoziation mit Leistungsmerkmalen untersucht.

Für die Identifizierung von Muskelfasertypen wurden drei Schweinerassen, Pietrain, Duroc und Mongcai sowie zwei Kreuzungen Duroc x Pietrain (DUPI) und Duroc x Berliner Miniaturschwein (DUMI) untersucht. Aus der Gruppe der Tiere der kommerziellen Rassen Pietrain und Duroc wurden neun unverwandte Tiere beprobt. Aus 598 Proben der F2-DUPI und 420 Proben der F2-DUMI wurden sechs divergente Vollgeschwistergruppen aus jeder Kreuzung ausgewählt, nach Geschlechtern balanziert, welche die extremen Werte für die Kotelettfläche repräsentieren.

Tiere von zwei kommerziellen Rassen und einer nativen Rasse wurden auf Unterschiede hinsichtlich von Merkmalen, die in Beziehung zu Muskularität und Fleischqualität stehen, untersucht. In den beiden kommerziellen Rassen Duroc und Pietrain wurde das Vorhandensein von MyHC Typ IIb in mehr als der Hälfte aller MyHC Transkripte gefunden (59,7 und 65,4 %). Im Hinblick auf die langsamen Fasern hatten die Tiere der Kreuzung DUMI einen signifikant höheren Anteil an MyHCI Isoformen als die anderen Rassen, mit Ausnahme der Mongcai. Das native Schwein Mongcai zeigte die langsamsten MyHC IIb Transkripte jedoch mit dem höchsten Anteil an anderen Isoformen im Vergleich zu den anderen Rassen und Kreuzungen. Aufgrund des genetischen Hintergrunds wurden höhere Prozentsätze der Muskelfläche bei den DUPI Tieren als bei den DUMI Tieren gefunden. Dies änderte jedoch nicht die Veränderung der Ausrichtung der Zusammensetzung der Fasertypen. Die verschiedenen Vollgeschwistergruppen in der DUPI und DUMI Population unterschieden sich signifikant ($P < 0,05$) in der relativen Anhäufung der MyHC Typ IIb Transkripte. In der DUPI Population wurden ähnliche Transkriptionslevel von MyHC I in beiden, den großen und den kleinen Muskeln gefunden. In DUMI waren diese Beobachtungen auch statistisch abzusichern. Durch ATPase Färbung nach der Säure Präinkubation (pH 4,6) konnten drei Fasertypen unterschieden werden. Die Korrelationen zwischen diesen Phänotypen lagen im Bereich zwischen 0,53 und 0,72 und waren signifikant ($P < 0,05$)

für die korrespondierenden Paare von Muskelfasern, welche durch Histochemie und Trankriptmenge der MyHC Isoformen charakterisiert wurden.

Im Mikroarray Experiment wurden Proben von vier verschiedenen Vollgeschwistergruppen der F2-DUPI Tiere verwendet. Diese Tiere waren im Hinblick auf die Kotelettfläche (groß im Vergleich zu klein) unterschiedlich, jedoch ähnlich bezüglich Alter, Schlachtgewicht und Schlachtkörpergewicht. Bei drei Genen (FTH1, FHL1C und RAMP1) wurde eine hohe Expression bei den Tieren mit der kleinen Muskelfläche gefunden, diese standen in Bezug zu der zellularen Kommunikation und der Regulation des Stoffwechsels. Dies wurde bei zwei Genen (FTH1 und FHL1C) in einer Real-Time RT-PCR bestätigt. Das TNNC1 Gen wurde aufgrund des Unterschieds der Faltung (1.8) in einer vorhergehenden Hybridisierung (QIAGEN Array) zwischen der kommerziellen Rasse Pietrain und der nativen Rasse Mongcai ausgewählt. Die Expressionslevel der RNAs zwischen den Schweinen war sehr weit gestreut, folgte jedoch dem Trend, dass TNNC1 hoch exprimiert ($P < 0{,}01$) in Pietrain verglichen mit Mongcai ist. Als Ergebnis wurde ein durchschnittlicher Wert der Faltungsänderung von 6.0 errechnet und daher festgestellt, dass TNNC1 ein höher reguliertes Gen bei Tieren der Rasse Pietrain ist. Neben der Auswahl von Genen aufgrund der Ergebnisse der Mikroarrays wurden auch Kandidatengene aus der Literatur ausgewählt. Das Gen CKM wurde aufgrund seiner positionellen und der funktionellen Beziehung zum Skelettmuskel beim Schwein ausgewählt. In einer Real-Time RT-PCR wurde festgestellt, dass CKM eine geringere Transkriptmenge in Mongcai verglichen mit Pietrain hat. Das signifikante Verhältnis ($P < 0{,}001$) der relativen mRNA Level von CKM zwischen den beiden extremen Schweinerassen weist auf seine potentielle Rolle für Muskel- und Fleischqualität hin.

Die SNPs wurden bei allen Genen in der F2 DUPI gefunden. Im FTH1 Gen lagen die beiden SNPs in der 3'UTR. In dem anderen Gen, welches ausgehend von der Mikroarray Analyse gefunden wurde, FHL1C, konnte kein SNP in allen Exons und einigen betrachteten Introns gefunden werden. Im TNNC1 Gen wurden vier SNPs in der Intron Region gefunden. Aus diesen wurde der 716 SNP für die Genotypisierung ausgewählt. Ebenso wurde ein SNP in der Promotor Region des CKM Gens gefunden.

Bei der Assoziationsanalyse konnte für den SNP1 im FTH1 Gen kein Effekt auf eines der betrachteten Merkmale gefunden werden. Bei den Parametern des Körpergewichts war der FTH1-SNP2 mit dem Tiergewicht zum Zeitpunkt des Versuchs (72 Tage)

assoziiert. Weiterhin hatte dieser SNP einen Effekt auf das Wachstumsstadium zu dem die Tiere gewogen wurden, bevor sie in die Testboxen umgestallt wurden. Die gesamte tägliche Zunahme der Tiere, die den Genotypen G/G beeinflusste war signifikant höher als die der Genotypen A/A (611g im Vergleich zu 589g). Für Merkmale der Fleischqualität stand die Scherkraft signifikant in Beziehung zu den Genotypen, besonders zu den homozygoten Tieren ($P = 0,025$).

Bei der Kombination zweier SNPs zur Konstruktion der Haplotypen, konnten für einige Merkmale signifikante Verbindungen zu einem bestimmten Genotypen gefunden werden. Das Alter der Schweine zum Zeitpunkt des Schlachtens beispielsweise war signifikant assoziiert mit dem Haplotypen GT/GT und AC/AC. Die Haplotypen AC/AC und CG/GT beeinflussten ebenfalls ADG3 und ADG4, jedoch nicht die frühen Stadien der Entwicklung. Eine hohe tägliche Zunahme, besonders in der letzten Phase des Wachstums ergab für den Haplotypen CG/GT den niedrigsten Wert für FCR (2,52 kg) mit einem statistischen Level von $P = 0,026$. Zusätzlich wurde für die Fleischfarbe eine Assoziation mit Haplotypen gefunden, wobei sich für den Haplotypen GT/GT einen höherer Wert ergab als für den Haplotypen CG/GT (70,7 und 65,8). Verschiedene Haplotypen waren ebenfalls mit dem pH_{24} Wert im *Semimembranosus* Muskel assoziiert, mit dem niedrigsten pH-Wert bei Tieren mit dem Haplotypen CG/GT (5,55) und dem höchsten pH-Wert bei dem Haplotypen GT/GT (5,64).

Bezüglich des SNP im TNNC1 Gen wurde die Kotelettfläche stark von einem Wechsel der heterozygoten C/G (52,0 cm²) zu den homozygoten G/G (50,2 cm²) Genotypen beeinflusst ($P = 0,001$). Weitere Merkmale der Körperzusammensetzung einschließlich Magerfleischanteil, Lendengewicht und geschätzte Magerfleischanteil im Schlachtkörper, die ebenfalls in Verbindung zur Kotelettfläche stehen, waren mit diesem SNP signifikant assoziiert mit *P*-Werten von jeweils 0,009, 0,014 und 0,001. In der gleichen Tendenz mit der Kotelettfläche wurden die Unterschiede nur zwischen C/G und G/G Genotypen gefunden, jedoch nicht bei den C/C Tieren. In diesem Muskel waren die Werte der Leitfähigkeit zu beiden Messzeitpunkten 45 min und 24 h p.m. ebenfalls signifikant assoziiert mit einer Veränderung der Genotypen. Zusammenfassend kann man sagen, dass dieses Merkmal im *Semimembranosus* Muskel 24 h p.m. bei Tieren mit dem C/C Genotypen niedriger war als bei Tieren mit den Genotypen C/G und G/G (3,5 im Vergleich zu 5,1 und 4,9).

Generell kann festgestellt werden, dass der SNP im CKM Gen höher in Beziehung stand zu der Körperzusammensetzung, speziell den Speckmerkmalen und mehr als zu der täglichen Zunahme oder den Fleischqualitätsmerkmalen. Die Ergebnisse bestätigen die Idee, dass die Anlage des Rückenspecks in späteren Wachstumsstadien stark ansteigt. Schweine mit dem A/A Genotypen zeigen ein höheres Schlachtgewicht und später höhere Speckmerkmale, verglichen mit denen mit den Genotypen A/G und G/G. Auf der anderen Seite hatten Tiere mit dem Genotypen A/A signifikant größere Speckflächen als die mit den beiden anderen Genotypen A/G und G/G (17,3, 15,8 und 15,5). Die gleiche Tendenz des Unterschieds zwischen diesen Genotypen wurde ebenfalls für das Verhältnis Speck zu Fleisch ermittelt und die Speckdicke über der Kotelettfläche. Die Speckdicke an den Rippen 13/14 der heterozygoten Tiere war ähnlich der von homozygoten Schweinen, sie unterschied sich nur auf solche Weise, dass die A/A Tiere einen dickeren Speck hatten als die G/G Tiere (2,86 im Vergleich zu 2,52). Im Gegensatz dazu hatten die Tiere mit dem Genotypen A/A niedrigere geschätzte Magerfleischanteile und Konditionierungsanteile als solche mit den Genotypen A/G und G/G ($P < 0,01$).

Zusammenfassend lässt sich feststellen, dass durch die Analyse der selektierten Vollgeschwistergruppen, die speziell für das Merkmal der Kotelettfläche ausgewählt wurden, jedoch in verschiedenen anderen Faktoren, welche ebenfalls die Fasereigenschaften beeinflussen könnten, gleich waren, konnte in dieser Untersuchung zum ersten Mal gezeigt werden, dass IIb die mögliche wichtige Faser ist, welche zu einem Unterschied in große und kleine Kotelettflächen beim Schwein führen kann. Weiterhin bestätigte der Kandidatengenansatz zum Teil die unterschiedlich exprimierten Gene aus den Ergebnissen der Mikroarrays in den zwei extremen Muskelgruppen. Dies ergab die Identifizierung von Genen, die mit ökonomisch wichtigen Merkmalen für die Schweineproduktion assoziiert sind.

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Acknowledgements

My greatest appreciation and thanks go to Prof. Dr. Karl Schellander, director of the Animal Breeding and Husbandry group, Institute of Animal Science, University of Bonn for accepting me as a Ph.D student and giving me an opportunity to conduct scientific research in the institute. I am really grateful to his valuable advice, thoughful comments and enthusiastic supervision making it possible for me to finish my study.

I would like to thank Prof. Dr. Karl-Heinz Südekum, director of the Animal Nutrition group, Institute of Animal Science, University of Bonn for his willingness and assistance as co-supervisor of this study.

I would like to express my special thanks to PD. Dr. Klaus Wimmers and PD. Dr. Siriluck Wimmers-Ponsuksili from the Research Institute for the Biology of Farm Animals (FBN), Dummerstorf for their careful guidance, thoughtful comments and scientific advice during the whole period of my research in Germany.

Sincere thanks go to Dr. Danyel Jennen and Dr. Dawit Tesfaye for their kind cooperation. Thanks for their technical help in various aspects, valuable comments and making available all facilities for carrying out this work.

I wish to thank Dr. Ernst Tholen, Mr. Heiko Buschbell and Mr. Detert Brinkmann for their kindness and willingness to help in data analysis.

To my former colleages, who have helped me enthusiastically not only in lab work but also in social activities since my first day arriving in Germany, I would like to thank Dr. Ali Kpatcha Kadanga, Dr. Kumar Ganesh, Dr. Solomon Mamo, Dr. Eduard Muráni and Mrs Muraniova Muráni. I really miss the friendly atmosphere that we have ever had.

My sincere thanks go to other colleages at the Institute of Animal Science for helping me in one way or another to successfully accomplish this task, especially to Dr. Patcharin Krutmuang, Dr. Cailu Lin, Dr. Guisheng Liu, Dr. Naras Trakooljul, Dr. Siriwadej Chomdej, Dr. Korakot Nganvongpanit, Dr. Patricio Ponce, Mr. Chiriwath

Phatsara, Ms. Patama Thumdee, Mr. Ashraf El-Sayed, Ms. Saowaluck Yammuen-art, Ms. Anke Brings, Mr. Nasser Ghanem and Mr. Parinya Wilaiphan. To my Vietnamese friends, I would like to express my special thanks to Mrs. Nguyen Thi Kim Khang and others not only for their being my superb colleagues but also for many great things that they have shared with me during the years I lived far away from home.

I would like to thank all administrative members of the Institute of Animal Science, particularly Ms. Bianca Peters, Ms. Ulrike Schröter and Mrs. Nicole Diel for their kind help with all documents. Thanks also go to Mr. Peter Müller for his really useful help in computer technique, Ms. Elisabeth Jonas for helping me with the German version of the thesis and Ms. Nadine Leyer for offering me samples whenever I need.

To my home Cantho University, I owe a heavy debt of gratitude to Asso. Prof. Dr. Le Quang Minh, Dr. Le Viet Dung, Asso. Prof. Dr. Vo Van Son, Mr. Nguyen Minh Thong, Mrs. Tran Thi Phan and Mr. Nguyen Van Hon for providing me an opportunity to be in Bonn and also for helping me with many administrative documents. I would like to thank other colleages not mentioned here by names for their important role they have all played.

I wish to express my sincere thanks to the Federal Ministry of Education and Research Germany (BMBF) and the Ministry of Science and Technology, Vietnam (MOST) for their grant that prompted me to successfully complete this work.

To my family no words can satisfactorily express my deep feeling of gratitude. My deepest gratitude goes to my parents, my younger brother and my wife for their endless encouragement and inspiration in all my studies.

Finally, I am very much grateful to all people who have contributed in one way or another for the realization of this work.

Curriculum vitae

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1992 – 1997: Bachelor of Science in animal husbandry and veterinary medicine. Department of Animal Husbandry, College of Agriculture, Cantho University, Vietnam.

1999 – 2001: Master of Science with a major in biology (tropical livestock system). Department of Animal Science and Management, Swedish University of Agricultural Science, Sweden.

2003 – 2006: Ph.D student. Institute of Animal Science – Animal Breeding and Husbandry Group, University of Bonn, Germany.

3. Training courses

1) Anti-nutrients, feed evaluation and feeding standards, Hohenheim University (Germany) and the National Institute of Aniamal Husbandry, Vietnam (August, 2001).
2) Design and analysis of biological experiments, Chiang Mai University, Thailand (November, 2001).
3) Animal reproduction in the tropics: physiology, pathology and enhancement, University of Agriculture and Forestry, Vietnam (March, 2002).
4) Practical DNA microarray analysis, Ludig Maximilians Universität – München, May 9th – 12th, 2005.

4. Publications

1) Nguyen Thi Hong Nhan, **Nguyen Trong Ngu**, Nguyen Van Hon, 1997. Effect of *Sesbania grandiflora* or *Leucaena leucocephala* on intake, digestion and rumen environment of goats fed a basal diet of maize stover. In proceedings of "Leucaena-Adaptation, Quality and Farming Systems" workshop. The University of Queensland, Australia, February 1998.

2) Nguyen Thi Hong Nhan and Nguyen Van Hon, **Nguyen Trong Ngu**, 1999. Supplementing rice by-products with foliage of *Trichanthera gigantea* in diets of growing and lactating pigs and fattening ducks. Livestock Research for Rural Development (11) 3 http://www.cipav.org.co/lrrd/lrrd11/3/nhan113.htm.

3) Nguyen Van Hon, **Nguyen Trong Ngu**, Nguyen Thi Hong Nhan, 2000. Rumen environment and digestibility on rice straw alone or supplemented with native grass. In proceedings of National Seminar, workshop on Sustainable Livestock Production on local feed resources. Agricultural Publishing House, Ho Chi Minh City, Vietnam.

4) Nguyen Thi Hong Nhan, Nguyen Van Hon, **Nguyen Trong Ngu**, Nguyen Tien Von, Preston TR and Leng RA, 2001. Practical application of defaunation of cattle on farms in Vietnam: response of young cattle fed rice straw and grass to a single drench of groundnut oil. Asian-Australian Journal of Animal Sciences (14) 4: 485-490.

5) **Nguyen Trong Ngu**, Nguyen Van Hung 2003. Performance of goats fed market waste from cabbage species supplemented with cake with high or low fiber content. Sida/Sarec, UAF, the final national Seminar workshop on Sustainable Livestock Production on Local Feed Resources SAREC 1988-2002, Hue City, 25 – 28 March, 2003.

6) Nguyen Thi Hong Nhan, Nguyen Van Hon, **Nguyen Trong Ngu**, Nguyen Thi Thu Hong, Preston TR and Leng RA, 2003**.** Effect of drenching with cooking oil on performance of local "Yellow" cattle fed rice straw and cassava foliage. Livestock Research for Rural Development 15 (7). http://www.cipav.org.co/lrrd/lrrd15/7/nhan157.htm

7) Nguyen Thi Hong Nhan , **Nguyen Trong Ngu**, Vo Van Son , Preston TR and Leng RA, 2005. Effect of an oil drench on the growth rate of cattle fed diets of low or high digestibility; Workshop-seminar "Making better use of local feed resources" (Editors: Reg Preston and Brian Ogle) MEKARN-CTU, Cantho, 23-25 May, 2005. http://www.mekarn.org/proctu/nhan09.htm

8) **Ngu NT**, Ponsuksili S, Jennen D, Tesfaye D, Schellander K, Wimmers K, 2005. Identification of candidate genes in pig muscle using microarray. Vortragstagung der DGfZ und GfT am 21./22. September 2005, Berlin, Deutschland (Vortr)

9) **Nguyen Trong Ngu** and Inger Ledin, 2005. Effects of feeding wastes from Brassica species on growth of goats and pesticide/insecticide residues in goat meat. Asian-Australian Journal of Animal Sciences 18 (2): 197-202.

10) Wimmers K, **Ngu NT**, Muráni E, Schellander K and Ponsuksili S, 2006. Linkage and expression analysis to elucidate the genetic background of muscle structure and meat quality in the pig. Archives of Animal Breeding 49, special issue, 116-125.

11) **Ngu NT**, Jennen D, Ponsuksili S, Muráni E, Hoelker M, Tholen E, Juengst H, Tesfaye D, Schellander K & Wimmers K, 2006. Poster and selected abstract for oral presentation: Myosin heavy chain isoform transcript abundance and muscle fiber type distribution of *Longissimus dorsi* in high and low performing pigs and in different pig breeds. Presented at the 30th Inernational Conference on Animal Genetics, realized in Porto Seguro, Bahia, Brazil, from August 20th to 25th, 2006.

11) **Ngu NT**, Jennen D, Ponsuksili S, Griese J, Muráni E, Hoelker M, Tholen E, Juengst H, Tesfaye D, Schellander K & Wimmers K, 2006. Poster: Trait-dependent expression and association of ferritin heavy-chain (FTH1) with muscularity and meat quality in pigs. Presented at the 30th Inernational Conference on Animal Genetics, realized in Porto Seguro, Bahia, Brazil, from August 20th to 25th, 2006.

12) Wimmers K, **Ngu NT**, Jennen D, Tesfaye D, Muráni E, Schellander K and Ponsuksilli S. Muscle size-associated transcript abundance of myosin heavy chain isoforms in *Longissimus dorsi* muscle in different pig breeds. Submitted.
13) **Ngu NT**, Jennen D, Tesfaye D, Muráni E, Ponsuksilli S, Schellander K and Wimmers K. Trait-dependent expression and association of ferritin heavy-chain (FTH1) with muscularity and meat quality in pigs. Manuscript.
14) **Ngu NT**, Jennen D, Tesfaye D, Muráni E, Ponsuksilli S, Schellander K and Wimmers K. Detection of single nucleotide polymorphism (SNP) in troponin C (TNNC1) and creatine kinase muscle (CKM) and their association with body composition and meat quality traits in pigs. Manuscript.

5. Awards

ISAG Travel Bursary Award for the abstract: Myosin heavy chain isoform transcript abundance and muscle fiber type distribution of *Longissimus dorsi* in high and low performing pigs and in different pig breeds. ISAG Conference 2006, August 20th - 25th, Porto Seguro, Brazil.