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Genomic characterization and polymorphism analysis of genes involved in lipid- and energy metabolism in swine

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Chapter 1

General introduction

Introduction

Domesticated pigs are raised as a food animal and pork is one of the most widely eaten meats in the world today (Jiang & Rothschild 2007). Most consumers desire both leanness and palatability in pork. Intramuscular fat content (IMF) is a major determinant of meat palatability. Pork provides not only an excellent source of high quality of protein, but also a major source of dietary fatty acids including saturated, mono-unsaturated and poly-unsaturated fatty acids (SFA, MUFA and PUFA respectively). Fatty acid composition of pork is of great interest because of its implications for human health. Excessive intake of SFA, particularly myristic acids and palmitic acids, is often associated with a high risk of cardiovascular diseases (Williams 2000); while increased intake of MUFA and PUFA is favorable due to their cholesterol decreasing effect (Stewart *et al*. 2001; Lichtenstein 2006). Hence, the lipid-related traits namely fatness, IMF and fatty acid composition are very important pork quality traits. These traits exhibit medium to high heritabilities (Sellier 1998), which justify the investigation of their genetic basis.

In pigs, conventional selection methods based on phenotypes have been successful in reducing backfat thickness due to the ease of obtaining phenotypes on live animals and its relative high heritability. Nevertheless, it is necessary to decipher the molecular architecture of fatness traits in pigs because the use of marker-assisted selection is expected to yield genetic gain over traditional phenotypic selection and the study might help understand the genetic basis of human obesity and other related health problems. Genetic improvement of meat quality traits such as IMF and fatty acid composition is difficult to achieve through traditional selection methods due to the need for extensive and expensive measurements of such traits on slaughtered relatives. However, it is expected that knowledge of the underlying genes for these traits will greatly contribute to the efficiency of selection.

There are two generally accepted approaches: the genome-wide scan approach and the candidate gene approach to locate genes affecting quantitative traits, e.g. the lipid-related quality traits in pigs (Rothschild 2003). The genome-wide scan approach uses segregation analysis either within commercial populations or in crossbreed populations to map quantitative trait loci (QTL) with effect on the trait of interest. Further molecular dissection of QTL is required to identify gene(s) and mutation(s) underlying the QTL. The candidate

gene approach starts with the choice of suitable candidate genes that may plausibly play a relevant role in the development of a given trait. Thus, the selection of candidate genes mainly relies on prior knowledge about the function of potentially contributing genes and (or) knowledge of the physiological basis of the trait under investigation. Moreover, the selection process could be facilitated if some of the potentially important genes are located in QTL regions obtained in the genome-wide scan. Following the identification of polymorphisms, an association study is conducted to estimate effect of polymorphisms in the candidate genes on the trait under investigation.

The development of traits such as fatness, IMF and fatty acid composition is closely related to lipid- and energy metabolism. Genome scans have identified a large number of QTL affecting these traits in pigs. Accordingly, genes, which encode key enzymes or key regulators in lipid- and energy metabolism and (or) are located within relevant QTL regions, are logical choices in the candidate gene analysis for these traits.

Pathways of lipid metabolism

Fatty acid *de novo* biosynthesis

A fatty acid contains a long hydrocarbon chain and a terminal carboxylate group. In humans, fatty acids are predominantly formed in the liver, adipose tissue, and mammary glands during lactation. The basic unit for building fatty acids is acetyl-CoA, which is generated in mitochondria primarily from two sourealize
rces: the pyruvate dehydrogenase reaction and fatty acid oxidation. Because fatty acids are synthesized in the cytoplasm, acetyl-CoA needs to be transferred from mitochondria to the cytoplasm. The transfer of acetyl-CoA to the cytoplasm is realized by its transport form, citrate. In mitochondria, citrate is formed from acetyl-CoA and oxaloacetate by citrate synthase. When present at high levels, citrate is transported to the cytoplasm where it is converted back to acetyl-CoA by ATP-citrate lyase (Tong 2005).

The synthesis of fatty acids starts with the carboxylation of acetyl-CoA to malonyl-CoA catalyzed by acetyl-CoA carboxylase (ACC), a biotin-dependent enzyme. This reaction is the first and committed step in fatty acid synthesis. ACC plays a key role in fatty acid biosynthesis and therefore, is highly regulated to control fatty acid metabolism (Berg *et al.* 2007). It can be switched off by phosphorylation of AMP-activated protein kinase (AMPK)

or activated by dephosphorylation of protein phosphatase 2A. Furthermore, it can be allosterically activated by citrate and inhibited by palmitoyl-CoA, and controlled by a variety of hormones (e.g. insulin, glucagon and epinephrine).

The following reaction involves the stepwise elongation of acetyl-CoA with two carbons each time (Berg *et al*. 2007). Malonyl-CoA is the source of the two carbons. Each elongation consists of four sequential steps: condensation, reduction, dehydration and reduction, all of which are catalyzed by one multifunctional enzyme complex - fatty acid synthase (FAS). In animals, fatty acid synthase is encoded by one gene (*FASN)*, but comprises seven catalytic sites (Smith 1994). The active enzyme system contains two identical FAS monomers. The primary product of FAS is palmitate.

Fatty acid elongation and desaturation

Additional fatty acid elongation and desaturation systems exist in mammals for generating longer saturated or unsaturated fatty acids. The elongation system is localized to the endoplasmic reticulum membrane. Unlike FAS for elongation, the system consists of several enzymes encoded by separate genes. It uses saturated and unsaturated fatty acyl-CoA as the substrates. However, the elongation reaction is similar to that catalyzed by FAS. It also uses malonyl-CoA as a donor to add two-carbon unit to the carboxyl ends of the substrates through four sequential steps (Fig. 1.1A).

The desaturation process that introduces double bonds in the long chain acyl-CoAs, also takes places in the endoplasmic reticulum. In mammals, Δ5, Δ6 and Δ9 desaturases are responsible for the synthesis of most of unsaturated fatty acids. All the three desaturases are membrane-bound and iron-containing proteins. Δ9 desaturase (also called stearoyl-CoA desaturase, SCD) catalyzes the last step of biosynthesis of monounsaturated fatty acids (MUFAs) from acetyl-CoA (Fig. 1.1B). This step introduces the first *cis*-double bond at the 9,10 position from the carboxyl end of saturated fatty acid substrates through oxidative reaction. Although the oxidation of the fatty acyl-CoAs also involves another two electron-transport proteins: NADH-cytochrome b5 reductase and cytochrome b5 (Fig. 1.2), SCD is the rate-limiting component in the reaction (Enoch *et al*. 1976; Nakamura & Nara 2004). The preferred substrates of SCD are palmitoyl-CoA and stearoyl-CoA, which are converted into palmitoleoyl-CoA (C16:1 cis-Δ9) and oleoyl-CoA (C18:1 cis-Δ9) respectively. These

two fatty acids (C16:1 and C18:1) are the major MUFAs of triglycerides, membrane phospholipids and cholesterol esters (Ntambi 1999; Nakamura & Nara 2004). Furthermore, various unsaturated fatty acids can be derived from palmitoleate and oleate. For example, oleate can either be elongated to a C20:1 cis-Δ11 fatty acid or be oxidized again to produce C18:2 cis-Δ6, Δ9 with double bonds. Likewise, palmitoleate can be enlongated to cis-vaccenate (C18:1 cis-Δ11).

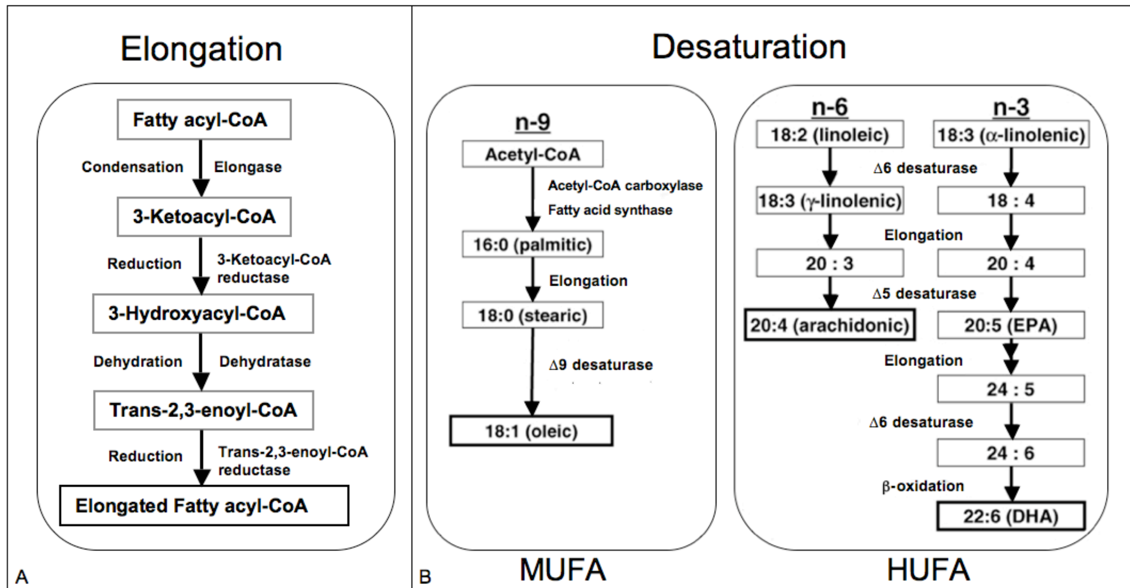

Figure 1.1 Elongation and desturation of fatty acids in mammals. Figure 1.1A shows the enzymes involved in two-carbon elongation of fatty acyl-CoA (Moon & Horton 2003). Figure 1.1B shows the synthesis of unsaturated fatty acids (Nakamura & Nara 2004). EPA-eicosapentaenoic acid, DHA-docosahexaenoic acid, MUFA-monounsaturated fatty acid, HUFA-highly unsaturated fatty acid.

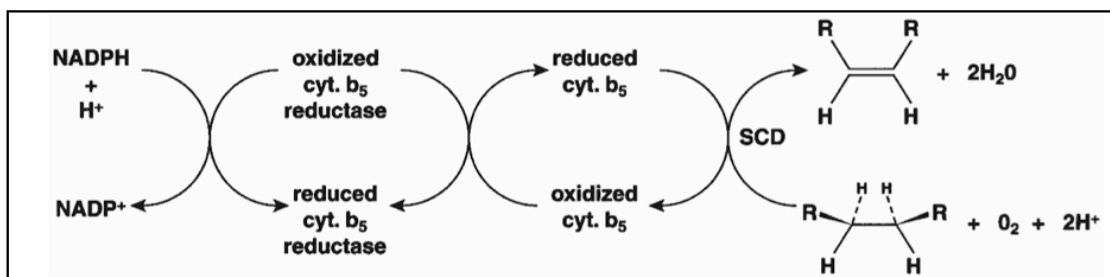

Figure 1.2 The pathway of electron transfer in the desaturation of fatty acids by stearoyl-CoA desaturase (Ntambi 1999).

Unlike insects and plants, mammals lack Δ12 and Δ15 desaturases, which can introduce double bonds at carbon atoms beyond C-9 in the fatty acid chain (Nakamura & Nara 2004). As a consequence, mammals cannot synthesize two essential fatty acids: linoleic acid (C18:2 cis-Δ9, Δ12) and alpha-linolenic acid (C18:3 cis-Δ9, Δ12, Δ15). Both must be provided in the diet of mammals. They are the starting points for the synthesis of highly unsaturated fatty acids (HUFAs), which involves a combination of elongation and desaturation reactions (Fig. 1.1B). The Δ5 and Δ6 desaturases, which introduce unsaturation at C-5 and C-6 respectively, are known to be the key enzymes in the desaturation reactions. In contrast to MUFAs and precursor PUFAs (linoleic acid and alpha-linolenic acid) that are readily incorporated into triacylglycerols, HUFAs are mainly stored in phospholipids, contributing to maintenance of biological membrane fluidity. Moreover, HUFAs play roles in the synthesis of eicosanoid hormones and the regulation of gene expression (Clarke 2000; Nakamura & Nara 2004).

Fatty acid degradation

Fatty acids are stored in the form of triacylglycerols primarily within adipocytes of adipose tissue. In response to the energy demand, fatty acids of the triacylglycerols can be mobilized for use by peripheral tissues. The utilization of fatty acids as fuel requires three stages of processing (Berg *et al*. 2007). In the first processing of lipolysis, triacylglycerols in adipose tissue are hydrolyzed by hormone-stimulated lipases to produce free fatty acids and glycerol. The released glycerol is absorbed in the liver where it either enters into the glycolytic or gluconeogenic pathway depending on the liver metabolic circumstances. The released fatty acids have very low solubility in blood plasma and are transported to energy-requiring tissues through their binding to serum albumin in the blood.

Second, the fatty acids must be activated in the cytoplasm at the energy-requiring tissues and transported into mitochondria for degradation. The activation of the fatty acids involves acyl-CoA synthetase, which catalyzes the formation of fatty acyl-CoA. The oxidation of fatty acids occurs in mitochondria. The transport of activated long-chain fatty acids into mitochondria is accomplished by a special system, which requires three proteins: carnitine acytransferase I (CPT1) and II (CPT2), and a translocase (Fig. 1.3); whereas activated medium-chain fatty acids can freely enter into mitochondria. The carnitine acytransferase I, which catalyzes the first step in the transport of long-chain fatty acids, is tightly regulated

by its physiological inhibitor malonyl-CoA. When fuel molecules are abundant, malonyl-CoA produced by acetyl-CoA carboxylase is present at a high level. It can inhibit CPT1 to prevent the entry of long-chain fatty acid into mitochondria for oxidation.

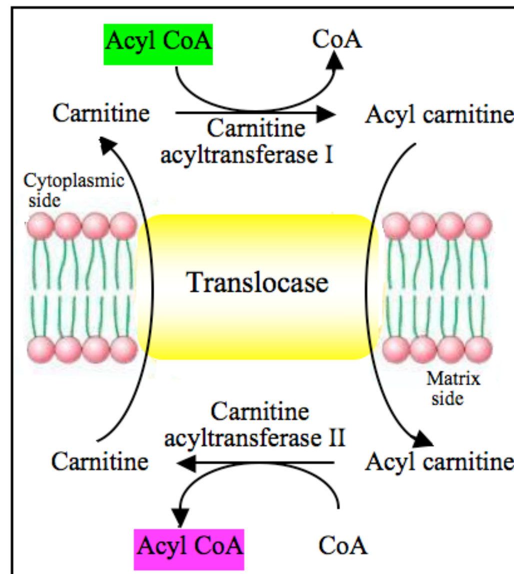

Figure 1.3 Transport of long chain fatty acyl-CoA across the mitochondrial membrane (Berg *et al*. 2007). Carnitine acyltransferase I located in the outer mitochondrial membrane converts the activated long-chain fatty acid (acyl CoA) into acyl carnitine in the presence of carnitine. The acyl carnitine is then shuttled across the inner mitochondrial membrane by a translocase. On the matrix side of the membrane, carnitine acyltransferase II converts acyl carnitine back to acyl CoA and carnitine. Finally the translocase returns carnitine to the cytoplasmic side in exchange for an incoming acyl carnitine.

Third, oxidation of the fatty acids occurs in mitochondria. The saturated fatty acids containing an even number of carbon atoms are oxidized through the β-oxidation pathway for the complete degradation. In the β-oxidation, 2-carbon unit is sequentially removed from the fatty acyl-CoA molecule. Finally, fatty acids are broken down into acetyl-CoA, which is then entered and processed in the citric acid cycle. However, additional isomerase and reductase are required for the degradation of unsaturated fatty acids. Fatty acids with an odd number of carbon atoms consist of a small portion of natural lipids. The final products from their oxidation contain not only acetyl-CoA but also propionyl-CoA. In this case, the

propionyl-CoA is converted into succinyl-CoA, which can then enter the citric acid cycle for further oxidation.

Fatty acid metabolism and energy balance

The hypothalamus and brainstem in the brain regulates energy balance in high animals through expression of orexigenic and anorexigenic neuropeptides that respond to hormonal signals (e.g. leptin and insulin) from peripheral tissues. The regulation network in the hypothalamus and brainstem is very complex and remains unclear so far. Recently, a growing body of evidence showed intermediates, particularly malonyl-CoA, of fatty acid metabolism in the hypothalamus participate in regulating energy homeostasis in the network (Dowell *et al*. 2005; Wolfgang & Lane 2006). Malonyl-CoA can be generated from different acetyl-CoA carboxylase isoforms (alpha and beta). Therefore, it serves different functions. Acetyl-CoA carboxylase alpha (ACACA) is expressed primarily in lipogenic tissues, e.g. the liver and adipose. In these tissues, malonyl-CoA is the substrate of fatty acid synthase (FAS) in *de novo* synthesis of long fatty acids. Acetyl-CoA carboxylase beta (ACACB) is highly expressed in skeletal and heart muscle. In these tissues, malonyl CoA acts as a regulator by regulating the entry of fatty acids into mitochondria. These tissues do not carry out fatty acid synthesis due to the lack of FAS enzyme. However, another enzyme - malonyl-CoA decarboxylase (MCD), which functions to move malonyl-CoA, is also highly expressed in these tissues.

In peripheral tissues, the cellular energy sensor - AMP-activated protein kinase (AMPK) - can regulate enzymes (ACC and MCD) involved in the formation and turnover of malonyl-CoA. Consequently, it leads to fluctuation of malonyl-CoA and directs the cell for fatty acid synthesis or oxidation (Xue & Kahn 2006). For example, when muscle stays in a low energy status, AMPK is activated due to an increase of 5' AMP relative to ATP. Following this, acetyl-CoA carboxylase beta (ACACB) is inactivated and MCD is activated through phosphorylation by AMPK. Both reactions result in a decrease in the concentration of malonyl-CoA. When malonyl-CoA is low, CPT1 is free from inhibition of malonyl-CoA and is activated to facilitate fatty acid transport into mitochondria for energy production. Therefore, malonyl-CoA as an intermediate of fatty acid synthesis plays an important role in the regulation of energy production through AMPK-malonyl-CoA-CPT1 pathway.

In the hypothalamus, malonyl-CoA serves as a modulator of food intake and energy expenditure (Wolfgang & Lane 2006). The evidence has come from several aspects. First, it has been found that the enzymes (AMPK, ACC, MCD and FAS) involved in the formation and turnover of malonyl-CoA are present in the hypothalamus. Second, inhibition of FAS by C75 in central nervous system, resulting in an increase of hypothalamic malonyl-CoA, led to the suppression of food intake. Moreover, concomitant with the increase of malonyl-CoA, expression of hypothalamic orexigenic neuropeptides was down-regulated and expression of anorexigenic neuropeptides was up-regulated (Loftus *et al*. 2000). Third, lowering malonyl-CoA by overexpression of MCD in the hypothalamus increased food intake and reversed the suppression of food intake by FAS inhibitor (Hu *et al*. 2005a; He *et al*. 2006). AMPK is the cellular energy gauge in the hypothalamus and can modulate the feeding behavior. The hypothalamic malonyl-CoA concentration appeared to be regulated by AMPK-ACC pathway (Dowell *et al*. 2005). A recent study about brain-specific carnitine palmitoyltransferase-1C (CPT1C) suggested that CPT1C could bind malonyl-CoA and that the disruption of CPT1C in mice resulted in decreased food intake and body weight (Wolfgang *et al*. 2006). It was speculated that CPT1C could be a downstream target of malonyl-CoA to convey 'malonyl-CoA signal' in hypothalamus (Wolfgang *et al*. 2006; Dai *et al*. 2007). All these studies support that the fluctuation of the hypothalamic malonyl-CoA leads to the regulation of energy metabolism.

Molecular genetics of the lipid-related quality traits in swine

Traits such as fatness, IMF and fatty acid composition are quantitative and their phenotypic values show continuous characteristics. Both genetic and environmental factors underlie these quantitative traits. Analysis of genetic architecture of these traits is carried out through the genome scan approach and the candidate gene approach, both of which aim at locating genes that affect the traits of interest.

Using molecular markers, mostly microsatellites, the genome-wide approach identified hundreds of quantitative trait loci (QTL) on porcine chromosomes during the last decade. The QTL for traits of interest can be acquired from Pig QTL database (PigQTLDB), which was established to integrate available pig QTL from public source (Hu *et al*. 2005b). To date, about 450 QTL for fatness traits measured at different locations have been reported

using different experimental and commercial populations (http://www.animalgenome.org/cgi-bin/QTLdb/SS/summary). These QTL are mainly located on SSC1, 2, 4, 5, 6, 7, 13 and X. Among them, SSC1, 7 and X are especially of most importance for fatness traits. Furthermore, it is found that the consistent QTL for fatness appear between 50 cM - 90 cM on SSC7 from independent observations (Meidtner 2007).

To date, a total of 23 QTL have been recorded for intramuscular fat content (IMF, Table 1.1). These QTL are located on nine porcine chromosomes including SSC1, 2, 4, 6, 7, 9, 13, 15 and X. SSC6 that harbors about half of the detected QTL for IMF, appears to be the important chromosome for this trait. Notably, within an interval of 24 cM from SW1355 to SW917 on SSC6, QTL for IMF have been observed five times.

Table 1.1 QTL for intramuscular fat content reported in different pig populations.

SSC	QTL span in cM (QTL center in cM)	Upper –lower significant marker (Peak)	Animals	Publications
1	16		Duroc × Large White	(Sanchez *et al.* 2007)
2	99		Large White × Meishan	(Qu *et al.* 2002)
4	56	AFABPMS	Large White × Dutch Landrace	(Gerbens *et al.* 2001)
4	4.1 - 69.6 (65)		Meishan × Dutch pigs	(de Koning *et al.* 1999)
4	62.3 - 72 (69.6)	S0217	Meishan × Dutch pigs	(Rattink *et al.* 2000)
4	105.8 - 121 (112)	SW445 - S0161	Large White × Meishan	(Su *et al.* 2004)
6	9.5 - 41.5 (29.2)	SW2535 - SW1841 (SW1353)	Meishan × Dutch pigs	(de Koning *et al.* 2000)
6	0 - 153 (65)		Large White × Dutch Landrace	(Gerbens *et al.* 2001)
6	83.3 - 90.7 (88.7)	SW1355 - SW1823	Duroc × Norwegian Landrace	(Szyda *et al.* 2003)
6	83.3 - 105.2 (97)	SW1355 - S0228 (SW2173)	Commercial line cross	(Grindflek *et al.* 2001)
6	90.7 - 105.2 (98.7)	Sw1823 - S0228	Duroc × Norwegian Landrace	(Szyda *et al.* 2002)
6	89.3 - 107 (102)	SW316 - SW917 (S0003)	Meishan × Dutch pigs	(de Koning *et al.* 2000)
6	97 - 106.1 (102)	SW2173 - SW2098 (S0003)	Iberian × Landrace	(Ovilo *et al.* 2002a)
6	65 - 155.2 (107)	SWR1130 - SW1069 (SW917)	Iberian × Landrace	(Ovilo *et al.* 2002b)
6	121.1 - 149.8 (138)	SW1881 - SW322	Commercial line cross	(Mohrmann *et al.* 2006)
6	102 - 161.4 (143)		Meishan × Dutch pigs	(de Koning *et al.* 1999)
7	109.5 - 117.3 (113.4)	SW1083 - SWR773	Meishan × Duroc	(*et al.* 2006)
7	117.3	SWR773	Meishan × Duroc	(Sato *et al.* 2003)
9	4	SW983	Meishan × Duroc	(Sato *et al.* 2003)
13	52		Duroc × Large White	(Sanchez *et al.* 2007)
13	117.5	SW769	Meishan × Duroc	(Sato *et al.* 2003)
15	50		Duroc × Large White	(Sanchez *et al.* 2007)
X	55.4 - 87.4 (71.7)	SW2456 - SW1943 (SW1426)	Meishan × White line	(Harlizius *et al.* 2000)

Table 1.2 Reported QTL affecting fatty acid composition in backfat and muscle.

Traits[1]	SSC	QTL Span (QTL center in cM)	Upper - lower significant marker	Animals	Publications
Backfat					
	1	3 -16.3 (14.7)	SW1824 - SWR485	Japanese wild boar × Large White	(Nii *et al.* 2006)
MP	2	53.5 - 59.5 (54)	FSHB - SW942	Japanese wild boar × Large White	(Nii *et al.* 2006)
	15	76 - 81.1 (77.7)	SW1945 - SW2083	Japanese wild boar × Large White	(Nii *et al.* 2006)
SFA	9	61.5 - 83.3 (78.6)	SW940 - SW944	Japanese wild boar × Large White	(Nii *et al.* 2006)
	15	28.9 - 50.7 (45.3)	CHRI-4 - SW964	Japanese wild boar × Large White	(Nii *et al.* 2006)
MUFA	X	73	ACSL4 – SW1943	Iberian × Landrace	(Mercade *et al.* 2006)
PI	4	75		Iberian × Landrace	(Clop *et al.* 2003)
UI	5	34		Iberian × Landrace	(Clop *et al.* 2003)
DBI	4	73		Iberian × Landrace	(Clop *et al.* 2003)
	6	105		Iberian × Landrace	(Clop *et al.* 2003)
ACL	8	60.4 - 112.3 (82.8)	S0225	Iberian × Landrace	(Clop *et al.* 2003)
	12	8 - 27 (18)		Iberian × Landrace	(Munoz *et al.* 2007)
C14:0	4	75		Iberian × Landrace	(Clop *et al.* 2003)
	10	82		Iberian × Landrace	(Clop *et al.* 2003)
	18	43.8 - 45.2 (43.8)		Landrace × Yorkshire	(Lee *et al.* 2003)
	12	3 - 9 (11)		Iberian × Landrace	(Munoz *et al.* 2007)
C16:0	8	60.4 - 112.3 (82.8)	S0225	Iberian × Landrace	(Clop *et al.* 2003)
	15	28.9 - 50.7 (35.9)	CHRI-4 - SW964	Japanese wild boar × Large White	(Nii *et al.* 2006)
	12	9 - 32 (20)		Iberian × Landrace	(Munoz *et al.* 2007)
C16:1 n-9	1	3 - 16.3 (14.1)	SW1824 - SWR485	Japanese wild boar × Large White	(Nii *et al.* 2006)
	8	60.4 - 112.3 (82.8)	S0225	Iberian × Landrace	(Clop *et al.* 2003)
	9	61.5 - 83.3 (64.6)	SW940 - SW944	Japanese wild boar × Large White	(Nii *et al.* 2006)
	12	63 - 77 (68)		Iberian × Landrace	(Munoz *et al.* 2007)
C18:0	1	3 - 16.4 (11.3)	SW1824 - SWR485	Japanese wild boar × Large White	(Nii *et al.* 2006)
	4	0		Iberian × Landrace	(Perez-Enciso *et al.* 2000)
	9	61.5 - 83.3 (67.6)	SW940 - SW944	Japanese wild boar × Large White	(Nii *et al.* 2006)
	12	68 - 84 (75)		Iberian × Landrace	(Munoz *et al.* 2007)
C18:1 n-7	12	69 - 83 (76)		Iberian × Landrace	(Munoz *et al.* 2007)
C18:1 n-9	4	81		Iberian × Landrace	(Perez-Enciso *et al.* 2000)
	15	76 - 81.1 (77)	SW1945 - SW2083	Japanese wild boar × Large White	(Nii *et al.* 2006)
	X	73	ACSL4 - SW1943	Iberian × Landrace	(Mercade *et al.* 2006)
C18:3	12	6.6 - 62.8 (45.2)	GHMSPI	Iberian × Landrace	(Clop *et al.* 2003)
C18:2	4	75		Iberian × Landrace	(Clop *et al.* 2003)
	4	71 - 86 (79)		Iberian × Landrace	(Perez-Enciso *et al.* 2000)
	4	62.3 - 69.6 (63.5)	SW839 - SW1089	Japanese wild boar × Large White	(Nii *et al.* 2006)
	12	22 - 40 (34)		Iberian × Landrace	(Munoz *et al.* 2007)
C20:1 n-9	X	52		Iberian × Landrace	(Mercade *et al.* 2006)
C20:2 n-6	12	1 - 21 (1)		Iberian × Landrace	(Munoz *et al.* 2007)
Muscle					
CLC	14	65		Duroc × Large White	(Sanchez *et al.* 2007)
MUFA	10	41		Duroc × Large White	(Sanchez *et al.* 2007)
SFA	14	45		Duroc × Large White	(Sanchez *et al.* 2007)
C14:0	15	44		Duroc × Large White	(Sanchez *et al.* 2007)
C16:1 n-9	14	67		Duroc × Large White	(Sanchez *et al.* 2007)
C18:0	14	67		Duroc × Large White	(Sanchez *et al.* 2007)
C20:3	9	88		Duroc × Large White	(Sanchez *et al.* 2007)
C20:5	10	27		Duroc × Large White	(Sanchez *et al.* 2007)
C22:5	7	48		Duroc × Large White	(Sanchez *et al.* 2007)

[1]MP, melting point; SFA, saturated fatty acids [%]; MUFA, monounsaturated fatty acids [%]; CLC, chain length coefficient; PI, Peroxidability index; UI, unsaturated index; DBI, double bond index; ACL, average chain length; C14:0, Myristic acid [%]; C16:0, Palmitic acid [%]; C16:1 n-9, Palmitoleic acid [%]; C18:0, Stearic acid [%]; C18:1 n-7 Cis-vaccenic acid [%]; C18:1 n-9, Oleic acid [%]; C18:3, Linolenic acid [%];

C18:2, Linoleic acid [%]; C20:1, Gadoleic acid [%]; C20:2, Eicosadienoic acid; C20:3 [%], Di-homo γ linolenic acid [%]; C20:5, Eicosapentaenoic acid (EPA) [%]; C22:5, Docosapentaenoic acid [%].

Compared to backfat and intramuscular fat content, there are more technical difficulties in measuring fatty acid composition. So far, only seven studies have reported QTL mapping results (Table 1.2). Six of them recorded QTL for fatty acid composition measured in backfat. QTL were found on SSC1, 2, 4, 5, 6, 8, 10, 12, 15, 18 and X. Comparison of the results for fatty acid composition in backfat revealed that the same study detected several QTL for one fatty acid parameter (e.g. melting point) and that different studies reported different QTL for the same measurement (e.g. C16:0). However, several QTL located from 62 cM - 86 cM on SSC4 for C18:2 from different studies showed consistency between independent observations.

The single investigation of QTL for fatty acid composition measured in muscle has appeared very recently using a Duroc × Large White cross (Table 1.2). The findings in muscle are quite different from those in fat. For example, QTL detected for C16:1, C18:0 and SFA in muscle are all located on SSC14 whereas QTL for them in fat are located on SSC1, 4, 8, 9 and 15. It was suggested that considering the low genetic correlations between muscle and backfat characteristics, this discrepancy was expected (Sanchez *et al.* 2007).

As can be seen from above, numerous QTL have been identified for these important traits. The QTL are often mapped to chromosomal regions that are over 20 centiMorgan long (20 megabase pairs) and that could possibly harbor several hundred genes (Andersson & Georges 2004). Therefore, the subsequent identification of gene(s) and mutation(s) that underlie QTL (positional cloning) remains a great challenge. Thus far, there are only a few success stories in domesticated animals. The one in pigs is the identification of the regulatory mutation *IGF2* intron 3-G3072A for QTL for lipid deposition and muscle growth on SSC2p in crosses between Large White and Wild Boar or Piétrain (Jeon *et al.* 1999; Nezer *et al.* 1999; Van Laere *et al.* 2003).

It is possible to identify functional candidate genes that are located in the QTL regions based on the comparative mapping approach. In this case, these identified genes are called positional and functional candidates. Using the candidate gene approach, several genes

have been investigated for lipid deposition and profile in pigs. Porcine heart fatty acid binding protein gene (*H-FABP*) serves as a positional and functional candidate gene for the consistent QTL on SSC6 with effect on intramuscular fat content. Several studies showed polymorphisms in this gene are associated with intramuscular fat content (Gerbens *et al*. 1999; Nechtelberger *et al*. 2001; Arnyasi *et al*. 2006). Polymorphisms found in porcine gastric inhibitory polypeptide gene (*GIP*), acetyl-CoA carboxylase alpha gene (*ACACA*) (Munoz *et al*. 2007) and acyl-CoA synthetase long-chain 4 gene (*ACSL4*) (Mercade *et al*. 2006) were found to show association with the concentration of different fatty acids measured in backfat. These associated polymorphisms are very unlikely to be causative because they seem to have no functional significance.

In conclusion, application of molecular methods has resulted in the identification of numerous chromosomal regions with QTL and several candidate genes associated with lipid deposition and profile. However, these identified regions with QTL always span tens of map units, or hundreds of genes. In order to make use of these QTL in the breeding program, the identification of important polymorphisms, which are responsible for the observed effect, is needed by using candidate gene approach. Nonetheless, lipid deposition and fatty acid composition in pigs are very complex traits that are likely to be controlled by many genes. For these reasons, it is necessary to evaluate new candidate genes for these traits.

Candidate genes

Overview of selected candidate genes

Backfat and intramuscular fat are the consequences of lipid deposition in adipose tissue and muscle respectively, indicating that lipid metabolism plays an important role in their development. Different lipid metabolic pathways, therefore, are also involved in the determination of fatty acid composition in backfat and intramuscular fat. Thus, it is logical to analyze candidate genes involved in lipid metabolism for backfat thickness, IMF and fatty acid composition in pigs.

As fatty acids are the building blocks from which lipids are made, fatty acid metabolism is the central part of lipid metabolism. Therefore, genes encoding key enzymes or key regulators in three main pathways of fatty acid metabolism namely fatty acid *de novo*

biosynthesis (*ACACA*), fatty acid desaturation (*FADS1*, *FADS2*, *FADS3*, *SCD1* and *SCD5*) and fatty acid oxidation (*ACACB*, *MLYCD*, *CPT1A*, *CPT1B* and *CPT2*) are the promising candidates for fatness, IMF and fatty acid composition (Fig. 1.4). AMPK plays a critical role in the regulation of energy balance. For this reason, *PRKAA2* encoding AMPK alpha 2 catalytic subunit is considered another interesting candidate for fatness and IMF traits in this study. Furthermore, most of the selected candidate genes are located in QTL regions reported for the backfat thickness, IMF or fatty acid composition.

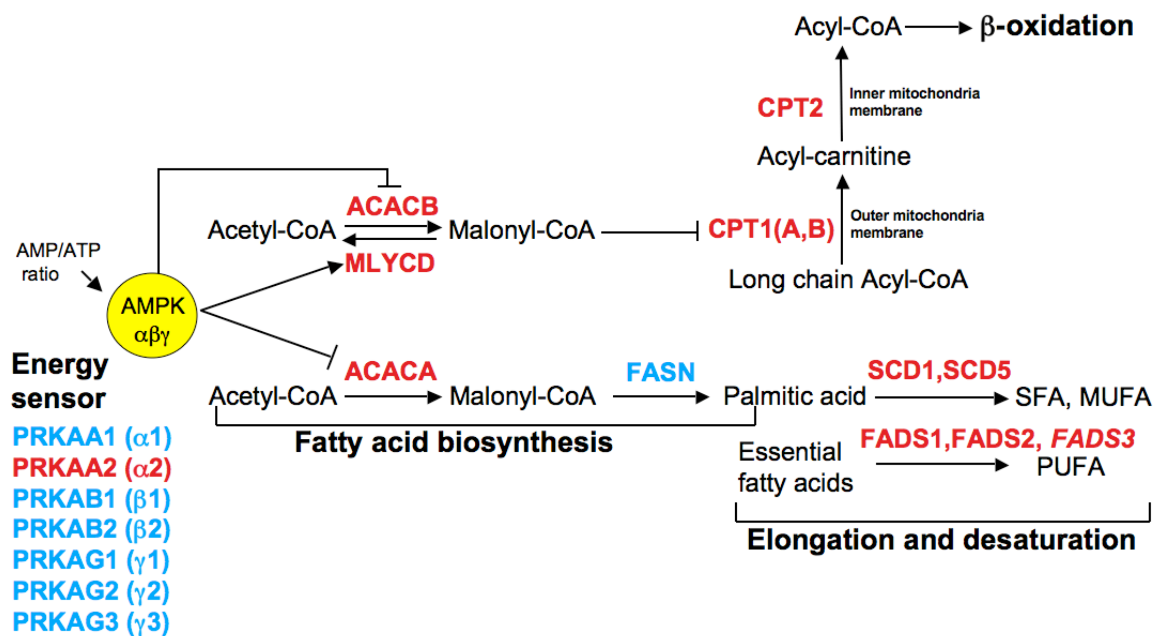

Figure 1.4 Genes encoding key enzymes or key regulators in lipid- and energy metabolism. Twelve genes highlighted in red were analyzed in this study.

Candidate gene involved in energy metabolism - AMPK alpha 2 catalytic subunit gene (*PRKAA2*)

The AMP-activated protein kinase (AMPK) is a serine/threonine kinase that is evolutionarily conserved from yeast to mammals and functions as a sensor of cellular energy status (Xue & Kahn 2006). AMPK is activated by a large variety of physiological and pathological stresses that deplete cellular ATP and increase AMP levels, including glucose deprivation, hypoxia, ischemia, oxidative stress, exercise and muscle contract. Its activation is mediated through allosteric activation due to AMP binding and/or AMP-facilitated phosphorylation. AMPK activation leads to phosphorylation of multiple

downstream targets switching off anabolic pathways (e.g. fatty acid and sterol synthesis) that consume ATP and switching on catabolic pathways (e.g. fatty acid oxidation) that generate ATP. The downstream targets include various key enzymes, such as acetyl-CoA carboxylase (ACC) (Fig. 1.4) and beta-hydroxy beta-methylglutaryl-CoA reductase (HMGCR), which are known to play critical roles in *de novo* biosynthesis of fatty acids and cholesterol respectively. Recent findings suggested AMPK in the hypothalamus mediates the effect of leptin on food intake and body weight (Minokoshi *et al*. 2004). Therefore, AMPK also plays an important role in regulating energy balance of the whole body.

AMPK is a heterotrimer comprising an α catalytic subunit and two regulatory subunits, β and γ. In mammals, each subunit has different isoforms that are encoded by separate genes (Fig. 1.4). There are two isoforms of the α subunit: α1 and α2 (Stapleton *et al*. 1996), three of the γ subunit: γ1, γ2 and γ3 (Cheung *et al*. 2000) and two of the β subunit: β1 and β2 (Thornton *et al*. 1998). Therefore, there are 12 theoretically possible AMPK (αβγ) complexes. The study about isoform compositions of AMPK complex indicated that three complexes (α2β2γ1>>α2β2γ3≥α1β2γ1) were detected in human *vastus lateralis* muscle (Wojtaszewski *et al*. 2005; Jorgensen *et al*. 2006).

In pigs, several of seven distinct genes encoding AMPK isoform α1, α2, β1, β2, γ1, γ2 and γ3 have been investigated. A mutation (known as *RN*$^-$) identified in porcine *PRKAG3* encoding γ3 subunit on porcine chromosome 15 (SSC15, Milan *et al*. 2000) was found to have detrimental effects on the technological yield of cured cooked ham, but positive effects on lean meat content (Le Roy *et al*. 2000). Subsequently, porcine *PRKAG1* (SSC5) and *PRKAG2* (SSC18) encoding γ1 and γ2 respectively were investigated to determine their effects on body composition (Demeure *et al*. 2004; Haberkern *et al*. 2004). The preliminary results failed to draw the final conclusion for the effect of the porcine *PRKAG1* on backfat thickness. This is because no polymorphism was detected in the analyzed pigs. The porcine *PRKAG2* was excluded as a candidate gene for body composition because it maps to a region on SSC18 where no QTL for such traits were reported (Demeure *et al*. 2004). The investigation of porcine *PRKAB1* (SSC14) and *PRKAB2* (SSC4) encoding β1 and β2 as candidate genes for meat quality traits was also initiated with their chromosome assignments (Fontanesi *et al*. 2003). The preliminary analysis of the SNP detected in one fragment of the porcine *PRKAB1* revealed no significant association. Although porcine

PRKAA2 has been proposed as a functional candidate gene for meat quality traits, neither mapping result nor polymorphisms were obtained (Fontanesi *et al*. 2003). Consequently, porcine *PRKAA1* and *PRKAA2* encoding α1 and α2 subunits need to be characterized in pigs. The porcine *PRKAA2* gene was analyzed in this study for the following reasons.

In contrast to the α1 isoform showing a wide expression pattern, the α2 subunit is highly expressed in the skeletal muscle, heart and liver (Stapleton *et al*. 1996). In addition, distinct physiological roles of the α1 and α2 catalytic subunits were revealed using knockout mouse models. AMPK α1-/- mice had no apparent metabolic defect, whereas AMPK α2-/- mice showed insulin resistance and reduced muscle glycogen synthesis (Viollet *et al*. 2003). Furthermore, another knockout experiment showed that the presence of AMPK α2 not α1 was required for muscle glucose uptake stimulated by adenosine analogue 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside (AICAR) (Jorgensen *et al*. 2004). The short-term overexpression of a constitutively active form of AMPK α2 (AMPK-CA) in the liver led to a significant decrease in blood glucose level, which subsequently resulted in an increase in hepatic lipid utilization and a decrease in white adipose mass (Foretz *et al*. 2005). The subcutaneous injection of AICAR significantly elevated α2 AMPK activity and the increased activity of α2 AMPK led to an acute reduction of protein synthesis in resting rat muscle through the mTOR signaling pathway (Bolster *et al*. 2002). The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription. The activation of α2 AMPK by leptin stimulated fatty acid oxidation in muscle (Minokoshi *et al*. 2002). The bidirectional changes in hypothalamic α2 AMPK were sufficient to regulate food intake and body weight (Minokoshi *et al*. 2004). In conclusion, α2 AMPK regulates glucose and fatty acid metabolism, and protein synthesis in peripheral tissues as well as energy intake and body weight.

Human *PRKAA2* was reported to be located at the type 2 diabetes locus on HSA1 (1p36-32) in a Japanese population (Horikoshi *et al*. 2006). Several association studies in different human populations were performed to examine the influence of *PRKAA2* on insulin resistance and susceptibility to type 2 diabetes (Horikoshi *et al*. 2006; Sun *et al*. 2006; Keshavarz *et al*. 2008). However, inconsistency was observed among their association results about the effect of *PRKAA2* on insulin resistance and susceptibility to type 2

diabetes. Additionally, one association study showed that several variants and a haplotype in the human *PRKAA2* have an effect on serum LDL-cholesterol and total cholesterol levels (Spencer-Jones *et al*. 2006).

Based on the critical roles of AMPK containing α2 (α2 AMPK) in peripheral tissues and the hypothalamus, *PRKAA2* was considered a functional candidate for meat quality and body composition traits in pigs. Moreover, the human and pig comparative map (Goureau *et al*. 1996) allowed the prediction that porcine *PRKAA2* is located on SSC6. Its location on SSC6 also made it a promising positional candidate for the reported QTL for intramuscular fat content, backfat thickness or loin muscle area (Ovilo *et al*. 2002b; Mohrmann *et al*. 2006; Edwards *et al*. 2008).

Candidate genes involved in fatty acid metabolism

Acetyl-CoA carboxylase genes (ACACA and ACACB)

There are alpha and beta acetyl CoA carboxylase isoforms in mammals, which are encoded by separate genes *ACACA* and *ACACB* respectively (Barber *et al*. 2005; Tong 2005). *ACACA* is highly expressed in lipogenic tissues (adipose and liver) and is involved in *de novo* fatty acid biosynthesis, while *ACACB* is predominantly expressed in skeletal muscle and heart and regulates fatty acid oxidation in mitochondria via its product, malony-CoA.

Mutant mice lacking *ACACA* are embryonically lethal, indicating that the *de novo* synthesis is very critical for development (Abu-Elheiga *et al*. 2005). Moreover, mice with live-specific deletion of *ACACA* are viable, but have 40-70 % less triglyceride accumulation in livers compared to the wide type mice, indicating that *ACACA* is also involved in energy metabolism (Mao *et al*. 2006). The recent work of Munoz *et al*. (2007) identified a QTL on SSC12 affecting the percentages of palmitoleic (C16:1 n-7), stearic (C18:0) and vaccenic (C18:1 n-7) acids in backfat and tested *ACACA* as a possible positional candidate gene for the QTL in an Iberian × Landrace cross. Two synonymous SNPs in *ACACA* were found to be associated with the percentages of these three fatty acids.

In contrast to ACACA, acetyl CoA carboxylase beta (ACACB) isoform has distinct roles in fatty acid metabolism. Findings in several knockout experiments in mice have consistently demonstrated that ACACB is a key regulator of mitochondrial fat oxidation (Abu-Elheiga

et al. 2001; Oh *et al*. 2005b; Choi *et al*. 2007). Mice lacking *ACACB* were characterized by no health problems, continuous fatty acid oxidation and reduced fat storage, and were also protected against diet-induced obesity and diabetes. Interestingly, a QTL with effect on the SFA content in meat in a Duroc × Large White cross was found on SSC14 (45 cM) in the proximity of porcine *ACACB* (Sanchez *et al*. 2007).

Malonyl-CoA decarboxylase gene (MLYCD)

Malonyl-CoA decarboxylase (MCD) catalyzes the degradation of malony-CoA to acetyl-CoA. The enzyme affects lipid partitioning because malony-CoA is the immediate precursor for *de novo* synthesis of fatty acids as well as a potent inhibitor of the CPT1 enzymes regulating fatty acid oxidation. The encoding gene (*MLYCD*) is highly expressed in the skeletal muscle, heart, kidney and pancreas, to a less extent in brain. Overexpression of *MLYCD* in the livers of rats ameliorates whole-animal and muscle insulin resistance induced by high-fat feeding (An *et al*. 2004). Thus, like *ACACB*, *MLYCD* could be another attractive antidiabetic drug target. MCD deficiency in humans is an autosomal recessive disorder characterized by malonic aciduria, developmental delay, seizure disorder, hypoglycemia, and cardiomyopathy. Based on the human and pig comparative map (Goureau *et al*. 1996), porcine *MLYCD* was predicted to lie on SSC6p where an imprinted QTL for IMF was identified in an experimental population of Chinese Meishan × Dutch pig lines (de Koning *et al*. 2000). The functional and positional arguments do not preclude *MLYCD* from being the candidate gene for this QTL. However, imprinting has not been reported for this gene in humans or other species.

Carnitine palmitoyltransferase genes (CPT1A, CPT1B and CPT2)

The ß-oxidation of long-chain fatty acids in mitochondria is a major source of energy, and the carnitine palmitoyltransferase (CPT) enzyme system facilitates the transport of such fatty acids into the mitochondria, which includes CPT1 and CPT2. There are three different CPT1 isozymes: CPT1A (liver-type), CPT1B (muscle-type) and CPT1C (brain-type), encoded by separate genes *CPT1A*, *CPT1B* and *CPT1C* respectively. *CPT1A* is mainly expressed in liver, and also in other tissues such as pancreas, kidney, brain and lung; *CPT1B* in brown adipose tissue, muscle and heart; and *CPT1C* only in the brain. Only one ubiquitous CPT II encoded by *CPT2* has been identified in mammals. The recent work by Sierra *et al*. (2008) revealed that in contrast to CPT1A and CPT1B, which are localized on the outer mitochondria membrane, CPT1C is localized in the endoplasmic reticulum of

neurons. Based on the subcellular localization, CPT1C was suggested to be involved in a biosynthetic pathway rather than a catabolic pathway (e.g. fatty acid oxidation in which CPT1A and CPT1B are implicated).

Consistent with their roles, deficiency of CPT (CPT1A, CPT1B and CPT2) results in mitochondrial fatty acid oxidation disorders in humans (Bonnefont *et al*. 1999; Yamazaki *et al*. 2008). Both *CPT1A* and *CPT1B* are located within genomic regions linked with obesity (Robitaille *et al*. 2007). Furthermore, sequence variants identified within *CPT1A* and *CPT1B* were found to show moderate associations with some obesity phenotypes. Human *CPT1A* is present at 68.36 Mb on human chromosome 11 (HSA11), approximately 7 Mb downstream of human *FADS* gene cluster on HSA11. In pigs, both are expected to be located on SSC2p downstream of *IGF2* where an imprinted QTL for fat deposition, muscle growth and heart size has been detected (Jeon *et al*. 1999; Nezer *et al*. 1999). Another distinct imprinted QTL only for backfat thickness was found approximately 57 cM from the *IGF2* region and no possible positional candidate genes was revealed (de Koning *et al*. 2000; Rattink *et al*. 2000). One QTL affecting both lean and, to a lesser extent, fat tissue weights, which did not exhibit any significant imprinting effect, was found near the IGF2 region in the INRA Meishan × Large White F_2 population (Milan *et al*. 2002). Apart from these QTL, several QTL affecting backfat thickness near the *IGF* locus on SSC2p were obtained using the Mendelian model by different studies (Knott *et al*. 1998; de Koning *et al*. 1999; Bidanel *et al*. 2001; Kim *et al*. 2005). The location of porcine *CPT1A* does not exclude it as a positional candidate for these Mendelian QTL. The comparative mapping places porcine *CPT1B* on SSC5. However, sequence comparison of porcine mRNA sequence (NM_001007191) against the preliminary sequences for SSC5 (http://pre.ensembl.org/Sus_scrofa/index.html) revealed no hit of this gene on the chromosome. Porcine *CPT2* is located in an interval (SW322-SW2053) of QTL for average backfat thickness on SSC6 (Mohrmann *et al*. 2006). Although porcine *CPT1C* was not included for analysis in this study, its role in energy homeostasis and its location on SSC6 makes it a promising functional and positional candidate gene for QTL (S0087- SW1129) affecting backfat thickness at 13 weeks and 17 weeks of age (Bidanel *et al*. 2001).

Fatty acid desaturase genes (FADS1, FADS2 and FADS3)

Fatty acid Δ5 (FADS1) and Δ6 (FADS2) desaturases are the rate-limiting enzymes in mammalian synthesis of long-chain polyunsaturated fatty acids. On human chromosome 11q12-q13.1, *FADS1* and *FADS2* are clustered with *FADS3* encoding fatty acid desaturase 3, whose function remains unknown (Marquardt *et al*. 2000). The *FADS* gene cluster is thought to have arisen evolutionarily from gene duplication based on their similar exon/intron organization. Three *FADS* genes in humans are highly expressed in the liver, heart and brain. Several studies in humans revealed that polymorphisms and haplotypes in the *FADS1-FADS2* gene cluster or the *FADS1-FADS2-FADS3* gene cluster show associations with levels of PUFA in serum phospholipids and in erythrocyte membranes (Schaeffer *et al*. 2006; Koletzko *et al*. 2008; Malerba *et al*. 2008; Rzehak *et al*. 2008). It has also been demonstrated that *FADS2* polymorphisms are associated with fatty acid profiles in the Japanese quail egg yolk, especially the n-6 and n-3 PUFAs (Khang *et al*. 2007). The above-mentioned studies strongly suggest that the *FADS* gene cluster is a promising candidate for fatty acid composition in pigs. However, no QTL affecting fatty acid composition have been identified in the proximity of the *FADS* gene cluster on SSC2.

Stearoyl-CoA desaturase genes (SCD1 and SCD5)

Stearoyl-CoA desaturase (SCD) is a crucial lipogenic enzyme necessary for the *de novo* biosynthesis of monounsaturated fatty acids in mammals. In humans (Wang *et al*. 2005) and cattle (Lengi & Corl 2007), two SCDs isoforms (SCD1 and SCD5) have been identified, whereas in mice four SCD isoforms, Scd1 through Scd4 exist (Tabor *et al*. 1998; Zheng *et al*. 2001; Miyazaki *et al*. 2003). Porcine *SCD1* has just been isolated recently (Ren *et al*. 2004b). Evidence for the existence of porcine orthologue of human and bovine *SCD5* was provided in this thesis.

Porcine *SCD1* maps to SSC14q27 (Ren *et al*. 2003). It lies downstream of the porcine *ACACB* on SSC14 based on the preliminary porcine genome sequence (http://pre.ensembl.org/Sus_scrofa/). Similar to mice lacking *ACACB*, mice deficient in *Scd1* display reduced body adiposity and increased insulin sensitivity, and are resistant to diet-induced weight gain (Ntambi *et al*. 2002). The consequence of *Scd1* deficiency is an activation of lipid oxidation in addition to reduced triglyceride synthesis and storage. In addition, a deficiency of *Scd1* ameliorates the obesity of ob/ob mice (mice lacking leptin because of a mutation in the ob gene) and completely corrects the hypometabolic

phenotypes of leptin deficiency (Cohen *et al.* 2002), indicating that down-regulation of *SCD1* is an important component of leptin's metabolic actions. Leptin is a 16-kD protein hormone derived from adipose tissue that plays a critical role in the regulation of body weight by inhibiting food intake and stimulating energy expenditure.

Based on its critical role in energy metabolism, *SCD1* has been investigated for fatness traits in pigs (Ren *et al.* 2004a). The analysis of a promoter SNP (AY487830: g.2228T>C) showed the lack of its association with backfat thickness in a pure Duroc population of 70 animals. The author suggested further association study using appropriate populations because only a small number of animals were used and it was insufficient to evaluate the effect of *SCD1* on fatness. In order to investigate the genetic factors that affect fatty acid composition of beef and milk, bovine *SCD1* has been chosen as one of the primer candidate genes in several studies. Results revealed that polymorphisms in *SCD1* explain some of the observed variation of fatty acid composition in beef (Taniguchi *et al.* 2004) and milk (Kgwatalala *et al.* 2007; Mele *et al.* 2007; Moioli *et al.* 2007; Milanesi *et al.* 2008; Schennink *et al.* 2008). In pigs, a suggestive QTL for the MUFA content in muscle in a Duroc × Large White cross was identified in a region where porcine *SCD1* was mapped (Sanchez *et al.* 2007). To date, no study has been performed to analyze *SCD1* for fatty acid composition in pigs.

SCD1 has been extensively studied since it was cloned for the first time in 1986 (Thiede *et al.* 1986), whereas *SCD5* has only been described recently. Both *SCD1* and *SCD5* are expressed in a variety of tissues. *SCD1* is highly expressed in lipogenic tissues such as adipose and liver, while *SCD5* in brain and pancreas (Wang *et al.* 2005; Lengi & Corl 2007, 2008). The human-pig comparative map allowed the assignment of porcine *SCD5* to the distal end of SSC8q. However, sequence comparison of the known porcine *SCD5* mRNA sequence (NM_001114278) against the preliminary sequence of SSC8 (http://pre.ensembl.org/Sus_scrofa/index.html) did not reveal its hit on the chromosome. It indicated that the preliminary sequence of SSC8 has yet to be completed. No QTL for the lipid-related traits have been reported at the position of *SCD5* so far.

Aims and outline of the thesis

Genes encoding key enzymes or key regulators in lipid- and energy metabolism may possibly affect the lipid-related traits in pigs. Hence, twelve such candidate genes have been selected and investigated in this thesis. Most of these genes have not been previously characterized. The aims of this thesis were:

1. Identification of porcine BACs containing candidate genes for the lipid-related traits using *in silico* BAC library screening approach
2. Genomic characterization of candidate genes for the lipid-related traits by BAC sequencing and semi-automatic annotation
3. Systematic screening for sequence variants
4. Direct evaluation of relationship between sequence variants in candidate genes and the lipid-related traits by association studies in different populations of pigs.

The twelve candidate genes have been selected primarily through literature review. *PRKAA2* encodes the alpha 2 catalytic subunit of the AMP-activated protein kinase (AMPK) that plays a very important role in the regulation of energy balance. This gene is considered a functional and positional candidate for the lipid-related traits. Chapter 2 describes the characterization of porcine *PRKAA2*: genomic structure, physical mapping, polymorphism detection and association study. The remaining eleven genes are those encoding key enzymes or key regulators in different pathways of lipid metabolism including fatty acid biosynthesis (*ACACA*), oxidation (*ACACB*, *CPT1A*, *CPT1B*, *CPT2* and *MLYCD*) and desaturation (*FADS1*, *FADS2*, *FADS3*, *SCD1* and *SCD5*). Most of them are localized to the regions harboring the relevant QTL reported. Genomic characterization and polymorphism analysis of these genes will be described in Chapter 3. The results presented in this study are discussed in Chapter 4 (General discussion). This chapter includes the discussion about selection of candidate genes, genomic characterization, sequence variants, association results and the candidate gene approach used in the study.

Chapter 2

Characterization of the porcine AMPK alpha 2 catalytic subunit gene (*PRKAA2*): genomic structure, polymorphism detection and association study

Abstract

AMP-activated protein kinase (AMPK), known as a key regulator of cellular energy homeostasis, plays an important role in regulation of glucose and lipid metabolism, and protein synthesis in mammals. The characterization of porcine *PRKAA2* encoding the alpha 2 catalytic subunit of AMPK is reported in this study. *PRKAA2* was assigned to porcine chromosome 6q by analysis of radiation hybrids (IMpRH panel), and its genomic structure was determined by BAC sequencing. *PRKAA2* spans more than 62 kb and consists of nine exons and eight introns. A total of 25 polymorphisms were identified by re-sequencing approximately 7 kb including all the exons, exon-intron boundaries, and 5' and 3' gene flanking regions using twelve founder animals of a Mangalitsa × Piétrain intercross. Neither of two single nucleotide polymorphisms (SNPs) found in the coding region causes an amino acid substitution. Two SNPs (NM_214266.1: c.236+142A>G and NM_214266.1: c.630C>T) in *PRKAA2* were genotyped in the Mangalitsa × Piétrain F_2 cross (n = 589) and two commercial populations: Piétrain (n = 1173) and German Landrace (n = 536), and evaluated for association with traits of interest (muscle development and fat deposition). Single SNP and haplotype analyses revealed weak associations between the *PRKAA2* genotypes and loin muscle area in the investigated populations.

Keywords association study, genomic characterization, pigs, *PRKAA2*

The AMP-activated protein kinase (AMPK) is a heterotrimeric protein comprising a catalytic subunit (α) and two regulatory subunits (β and γ), which plays an important role in the regulation of cellular energy balance as well as the whole body energy metabolism (reviewed by Xue & Kahn 2006). A mutation (known as *RN⁻*) identified in porcine *PRKAG3* encoding AMPK subunit isoform γ3 (Milan *et al.* 2000) was found to have a detrimental effect on the technological yield of cured-cooked ham, but a positive effect on lean meat content (Le Roy *et al.* 2000). There are two distinct genes encoding AMPK α isoforms: α1 (*PRKAA1*) and α2 (*PRKAA2*) (Hardie *et al.* 2003). Neither of them has been previously characterized in pigs. *PRKAA2* is of special interest due to the following evidence: (1) in contrast to the α1 isoform showing a wide expression pattern, α2 is highly expressed in the skeletal muscle, heart and liver (Stapleton *et al.* 1996); (2) AMPK $\alpha1^{-/-}$ mice have no apparent metabolic defect, whereas AMPK $\alpha2^{-/-}$ mice show insulin resistance and reduced muscle glycogen synthesis (Viollet *et al.* 2003); (3) activation of α2 AMPK by 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) suppresses protein synthesis in rat skeletal muscle (Bolster *et al.* 2002); (4) activation of α2 AMPK by leptin stimulates fatty acid oxidation in the muscle (Minokoshi *et al.* 2002); (5) the bidirectional changes in hypothalamic α2 AMPK are sufficient to regulate food intake and body weight (Minokoshi *et al.* 2004); (6) the short-term overexpression of a constitutively active form of AMPK α2 (AMPK-CA) in the liver causes a significant decrease in blood glucose level, which leads to an increase in hepatic lipid utilization, resulting in a decrease in white adipose mass (Foretz *et al.* 2005).

Taken together, these observations suggest the involvement of AMPK containing α2 in the regulation of glucose and lipid metabolism, and protein synthesis in peripheral tissues as well as in the regulation of energy intake and body weight, hence making *PRKAA2* encoding α2 subunit a candidate for muscle development and lipid deposition in pigs. The objectives of the present study were to determine genomic structure and chromosomal location of porcine *PRKAA2*, to identify polymorphisms and to investigate their possible associations with traits related to muscle development and fat deposition in different pig populations.

To determine the gene structure, the clone (189G19) from porcine CHORI-242 BAC library (http://bacpac.chori.org/porcine242.htm) was identified for *PRKAA2* based on

BLAST results of porcine BAC end sequences against human reference sequence available online at http://www.sanger.ac.uk/Projects/S_scrofa/BES.shtml. The presence of *PRKAA2* within this clone was further confirmed by colony PCR (Table 2.1). A shotgun approach (Retter *et al*. 2007) was performed to sequence the clone. Comparison of the known porcine mRNA sequence (NM_214266.1) with the sequence data assembled using the GAP4 program (Staden 1996) identified four genomic contigs containing *PRKAA2*, which have been deposited in GenBank under accession number EU853704. The comparison also revealed that *PRKAA2* contains nine exons and eight introns and covers at least 62 kb of genomic DNA (Fig. 2.1). All exon-intron junctions follow the GT-AG rule. The genomic structure is similar to that of human *PRKAA2* (Horikoshi *et al*. 2006).

The deduced protein sequence comprising 552 residues shares 98% identity with human AMPK α2 catalytic subunit sequence (NP_006243). The comparison of AMPK α2 subunit sequences from different species facilitated the identification of conserved domains in porcine AMPK α2 subunit. Likewise, porcine AMPK α2 contains an N-terminal catalytic domain, a middle auto-inhibitory domain and a C-terminal β/γ binding domain (Fig. 2.2). These domains are the structure feature of α catalytic subunit of AMPK (Hardie *et al*. 2003; Wong and Lodish 2006). Recently, Pang *et al*. (2007) narrowed the previously reported auto-inhibitory domain (α1: residues 313-392) to 23 amino acids (Fig. 2.2). The threonine residue at position 172 (Thr-172), which is phosphorylated for AMPK activation by a family of upstream kinases including LKB1, was also identified in the catalytic domain of porcine AMPK α2 subunit (Fig. 2.2). AMPK exists as a heterotrimer consisting of a catalytic α-subunit and two regulatory β- and γ- subunits. In the heterotrimer kinase complex structure, the C-terminus of β-subunit interacts with the C-terminal β/γ binding domain in α-subunit; whereas the direct binding of γ-subunit to α subunit involves not only the interaction site in β/γ binding domain of the α-subunit, but also the site in the catalytic domain (Wong and Lodish, 2006). There is no direct interaction between β- and γ- subunit. AMPK is activated by high intracellular AMP/ATP ratios. Its activation is initiated by the binding of two molecules of AMP to γ-subunit in the AMPK complex, and is followed by the phosphorylation of α subunit at Thr-172 site by the upstream kinases due to the binding of AMP to AMPK.

Table 2.1 Primers and PCR conditions used for colony PCR, physical mapping and re-sequencing.

Gene/Primer number[a]	Primer sequences (5'-3')	Product size (bp)	Primer binding region	Annealing temperature (Additives)
PRKAA2_4717up	GGCAATAGAAGGCTGAGTGC		5' flanking	
PRKAA2_4718dn	CCTAGCACCTGTAAACAACCTG	489	5' flanking	60°C (DMSO)
PRKAA2_4715up	CACCCACTTGTTTGGCATAA		5' flanking	
PRKAA2_4716dn	AATGGCGAGCATACATCCAT	513	5' flanking	60°C (DMSO)
PRKAA2_4654up	GGCTATTTTCTCCAAATCGTCAG		5' flanking	
PRKAA2_4655dn	CGAACAATCAGACCCAAGAGTTA	729	5' flanking	60°C
PRKAA2_4652up	TTGCCCTAGCTCACCGTAGT		5' flanking	
PRKAA2_4653dn	GAGAACCCGAAGGAGTGGAA	558	Promoter	60°C (DMSO)
PRKAA2_4272up	GCTGAGAAGCAGAAGCACGAC		Exon 1	
PRKAA2_4273dn	CACCTTGCCCAACAGTCCAGT	440	Intron 1	60°C (Q-solution)
PRKAA2_4101up	TTGGGGTTACAGTGGAAGGA		Intron 1	
PRKAA2_4102dn	GTATGACAAAGAAGAGGCAACG	441	Intron 2	57°C
PRKAA2_4439up	AGCAAGAATGGATATACTAGGTTGA		Intron 2	
PRKAA2_4440dn	TGTTTCAGGGAATCAGCAAGT	497	Intron 3	60°C
PRKAA2_4274up	CCCCAAATCTTCTTAATGCTGT		Intron 3	
PRKAA2_4275dn	GCTAGTATCCTTCTAAACCACCTTC	221	Intron 4	60°C
PRKAA2_4276up	TGAGGCTTTACAGACTTCAGGTT		Intron 4	
PRKAA2_4277dn	CCCGAATGAACAAAACATTACAA	244	Intron 5	60°C
PRKAA2_4103up	GAAGTCCTTGAAAGGCAATGTA		Intron 5	
PRKAA2_4104dn	GATCAGGCAATCAGTTAGTGGTA	424	Intron 6	57°C
PRKAA2_4267up	TATTTGGGACTTGCCAGAGC		Intron 6	
PRKAA2_4268dn	AAAGAACCAGTTGGGGGACT	321	Exon 7	58°C
PRKAA2_4269up	CCCCTCCTATGATGCTAACG		Exon 7	
PRKAA2_4270dn	TGATGCATGCTGAAACAGGT	781	Intron 7	58°C
PRKAA2_4107up	CTTTAGAGTAGAGGCACATCTGGA		Intron 7	
PRKAA2_4108dn	GATCCTATTCGCTTCACTTACACT	551	Intron 8	57°C
PRKAA2_4109up	TGCTTGTTAGGTTGTCCTTGCT		Intorn 8	
PRKAA2_4110dn	GAGTGTCCTCTGGAGATAGTTACG	486	3' UTR	60°C
PRKAA2_3827up	GTTGGATTCTGTCACTGCGGA		Exon 9	
PRKAA2_3828dn	CAAGCGACTCTTCGTTGATGGT	497	Exon 9	60°C
PRKAA2_4278up	TGTTGTTCATCTAAAACCGTGG		Exon 9	
PRKAA2_4279dn	CTCTTTACAGGGACTACCGACAT	607	Exon 9	60°C
PRKAA2_4719up	TCACCAAGCATGATTTGACAG		3' UTR	
PRKAA2_4720dn	GGATGGCTAGCAACCAAGAT	277	3' flanking	60°C
PRKAA2_4721up	TGGCCGACTGACTTAGCTTT		3' flanking	
PRKAA2_4722dn	CCAAACTGGCAAACAAGGAT	752	3' flanking	60°C

[a]Primers (4109up/4110dn) were used for genotyping the INRA-Minnesota porcine radiation hybrid panel and primers (3827up/3828dn) for colony PCR.

The PCR analysis (Table 2.1) of the INRA-Minnesota porcine radiation hybrid panel (IMpRH panel, Yerle *et al.* 1998) demonstrated that *PRKAA2* is closely linked to marker SW322 on SSC6q31-q35 with LOD score threshold of 11.41. This assignment agrees well with that expected by comparative mapping with the human *PRKAA2* gene on HSA1p31

(Beri *et al*. 1994), and also with that indicated by the location of the BAC CH242-189G19 containing *PRKAA2* on the Porcine Genome Physical BAC map (http://www.sanger.ac.uk/Projects/S_scrofa/mapping.shtml).

Figure 2.1 Genomic structure of the porcine *PRKAA2* gene, 25 identified polymorphisms (Table 2.2) and haplotype distribution in the parental (P), F_1 and F_2 generations of the M × P intercross. Three tag SNPs (1023, 1003 and 1024) highlighted with black boxes correspond to c.236+142A>G, c.630C>T and c.699T>C, respectively. Symbols 'D' and 'I' are designated as deletion and insertion of two nucleotides (CT), respectively.

Figure 2.2 Functional regions of porcine AMPK α2 subunit. The auto-inhibitory domain is indicated with symbol 'a'.

Animals from three different populations were used in this study. The first was a three-generation pedigree: a Mangalitsa × Piétrain intercross (M × P). The parental generation included two Mangalitsa boars, and 13 Piétrain sows that were all homozygous for the unfavorable allele T at the *RYR1* c.1843C>T polymorphism (Fujii *et al*. 1991). Five F_1 males and 18 F_1 females were used to produce 613 F_2 animals. Animals were fed *ad*

libitum. F_2 males were castrated. All F_2 pigs were slaughtered at body weight of 90-100 kg. A total of 42 phenotypes measuring meat quality and body composition were collected on the F_2 animals including fatty acid composition, intramuscular fat content (IMF), loin muscle area (LMA) and backfat thickness measurements (BFT). The other two were commercial populations: German Landrace (n = 536) slaughtered at 107.2 ± 2.7 kg within 2002-2005 and Piétrain (n = 1173) at 103.6 ± 2.1 kg within 2003-2006. The phenotypes recorded in both populations mainly included body composition traits. In addition, IMF was phenotyped in the Piétrain animals.

Screening for polymorphisms in *PRKAA2* was performed by PCR amplification and sequencing of the twelve parental animals of the M × P intercross (two Mangalitsas and ten Piétrains). Eighteen primer pairs (Table 2.1) were used covering all nine exons, exon-intron boundaries, and about 2 kb 5' and 1kb 3' gene flanking regions. In total, 25 polymorphisms were identified comprising 24 SNPs and one insertion/deletion polymorphism (Fig. 2.1 and Table 2.2). Four haplotypes (Fig. 2.1) were derived from the 25 polymorphisms in the parental generation of the M × P family using PHASE version 2.1 (Stephens *et al*. 2001; Stephens & Donnelly 2003). Fixed alleles in the two Mangalitsa founder boars were observed at all the 25 polymorphisms. Therefore, both of them were homozygous for haplotype 1 (HT1, Fig. 2.1). All the 25 polymorphisms were segregating within the ten Piétrain sows, and four haplotypes (HT1, HT2, HT3 and HT4) were present with a frequency of 0.38, 0.16, 0.42 and 0.04 respectively in this small sample. Three tag SNPs: NM_214266.1: c.236+142A>G, NM_214266.1: c.630C>T and NM_214266.1: c.699T>C were sufficient to distinguish between the four haplotypes (Fig. 2.1). The following analysis using these three SNPs revealed that HT4 was absent in the F_1 animals (Fig. 2.1). Therefore, only two SNPs (c.236+142A>G and c.630C>T) were genotyped in the F_2 population using PCR-RFLP (Table 2.3). Both SNPs were also investigated in the commercial Piétrain and German Landrace populations. However, Taqman SNP genotyping assays (Table 2.3) were used instead to increase the genotyping efficiency. Genotypes of the *RYR1* mutation (Fujii *et al*. 1991) in both the M × P F_2 cross and the Piétrain population were determined using TaqMan genotyping assay (Table 2.3). This mutation was not segregating in the German Landrace population based on a pilot experiment of about 200 animals for allele frequency estimation.

Table 2.2 Twenty-five polymorphisms found in the *PRKAA2* gene.

No.	Lab_id	Genomic reference sequence (EU853704)[a]	Coding DNA reference sequence (NM_214266.1)[a]	Region
1	1342	g.14519C>A	c.-1864C>A	5' end
2	1340	g.14760C>T	c.-1623C>T	5' end
3	1264	g.15293G>A	c.-1090G>A	5' end
4	1265	g.15337C>G	c.-1046C>G	5' end
5	1266	g.15476G>A	c.-907G>A	5' end
6	1267	g.15730C>T	c.-653C>T	5' end
7	1268	g.15886A>C	c.-497A>C	5' end
8	1461	g.15958C>T	c.-425C>T	5' end
9	1060	g.16543G>C	c.94+67G>C	Intron 1
10	1022	g.45837G>A	c.236+112G>A	Intron 2
11	1023	g.45867A>G	c.236+142A>G	Intron 2
12	1095	g.52668A>G	c.330+262A>G	Intron 3
13	1003	g.58022C>T	c.630C>T	Exon 6[b]
14	1024	g.58091C>T	c.699T>C	Exon 6[b]
15	1052	g.73495A>T	c.1293+30A>T	Intron 7
16	1053	g.73509G>A	c.1293+44G>A	Intron 7
17	1054	g.73510A>T	c.1293+45A>T	Intron 7
18	1055	g.73539G>A	c.1293+74G>A	Intron 7
19	1076	g.74712_74713del	c.1294-101_1294-100delCT	Intron 7
20	1025	g.74963A>G	c.1420+24A>G	Intron 8
21	1093	g.77454A>G	c.*545A>G	3' end
22	1494	g.77688A>T	c.*779A>T	3' end
23	1495	g.77689T>C	c.*780T>C	3' end
24	1338	g.77927C>T	c.*1018C>T	3' end
25	1339	g.78496G>A	c.*1587G>A	3' end

[a]Polymorphisms were described using standard nomenclature (den Dunnen & Antonarakis 2000).

[b]Two synonymous SNPs were found in exon 6.

Table 2.3 Genotyping methods used for the c.236+142A>G and c.630C>T SNPs in the *PRKAA2* gene and the C1843T polymorphism in the *RYR1* gene.

	Genotyping methods	
SNPs	PCR-RFLP (primers-enzyme)[a]	*Taqman* assay primers and MGB probes[b]
c.236+142A>G	4101up/4102dn CvikI-1	PRKAA2-1023F: TGTAAGTATTTTTGCATTTGATAATATAAAAGATGAAGTGT PRKAA2-1023R: GGCAACGTAAATTACATTTTTAGCTCTGA PRKAA2-1023A: CATTAATTCATTTA**A**CCTCC (VIC labeled) PRKAA2-1023G: CATTAATTCATTTA**G**CCTCC (FAM labeled)
c.630C>T	4103up/4104dn RsaI	PRKAA2-1003F: CTGCGGTGTTATTTTGTATGCTCTT PRKAA2-1003R: CCCCTCGGATCTTCTTAAACAATGT PRKAA2-1003C: ATGGGAGTGT**G**CCACAA (VIC labeled) PRKAA2-1003T: AATGGGAGTGT**A**CCACAA (FAM labeled)
c.1843C>T		RYR1-F: CCCTGTGTGTGTGCAATGG RYR1-R: GTTTGTCTGCAGCAGAAGCT RYR1-1120C: CCGTG**C**GCTCCAA (VIC labeled) RYR1-1120T: CCGTG**T**GCTCCAA (FAM-labeled)

[a]The primer sequences are shown in Table 2.1.

[b]Genotyping was carried out in ABI 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA).

The statistical analysis involved R language and environment (http://www.r-project.org/). A linear model was used to assess the effect of *PRKAA2* genotypes on the traits of interest, namely IMF, BFT and LMA. For the M × P F_2 population, the model included the fixed effects: father, mother, sex and genotype. For haplotype analysis, the genotype was represented as the copy number of the haplotype in question and was treated as a factor. Live weight at slaughtering was used as a covariate for BFT and LMA, and BFT for IMF. For each commercial population, in addition to the covariates described above, the model used contained fixed effects: performance testing station and genotype, and a random sire effect (due to more sires used in the commercial populations). The effect of sex was not included in the model because animals in each commercial population were of the same gender. A major effect of the *RYR1* c.1843C>T mutation on muscle mass and lipid deposition has been reported (Fujii *et al.* 1991). Therefore, the effect of the *RYR1* genotypes was also taken into account when analyzing the F_2 and Piétrain populations

where there was a high incidence of the *RYR1* mutation (0.51 and 0.43 respectively). Least squares means (± SE) for the individual SNPs and constructed haplotypes were determined based on their respective linear models.

The allele frequencies of the c.236+142A>G and c.630C>T SNPs, and the derived haplotype distribution are listed in Table 2.4. The c.630C>T SNP was not segregating in the German Landrace population. Therefore, no haplotype was constructed in this population. Both the c.236+142A>G and c.630C>T SNPs were segregating in the Piétrain population. For the reasons of technical difficulties with the c.236+142A>G SNP genotyping assay and large variation in the concentration of DNA samples placed in the plates, only 865 Piétrain animals that were successfully genotyped were used for haplotype construction. No deviation of the genotype distribution from Hardy-Weinberg equilibrium was observed at the segregating SNPs in the commercial populations.

Results from the association studies are presented in Table 2.4. There was no significant association detected between the *PRKAA2* SNPs or haplotypes and IMF or BFT in all the three populations under investigation. Therefore, Table 2.4 only shows the association results for LMA. Single SNP analysis revealed that both the c.236+142A>G and c.630C>T SNPs were significantly associated with LMA in the M × P F_2 population ($P < 0.05$). It was expected that HT1 and HT3 would show association with LMA in the M × P F_2 population because HT1 and HT3 were equivalent to the c.630C>T and c.236+142A>G SNPs respectively in this population (Fig. 2.1). The G allele at the c.236+142A>G SNP, the T at the c.630C>T SNP and their combination HT3 (G-T), which seemed to increase LMA (Table 2.4), were all derived from the Piétrain breed that is well known for exceptional muscularity.

Table 2.4 Association results between the SNPs or haplotypes in *PRKAA2* and loin muscle area in the M × P F_2 and two commercial pig populations.

SNP analysis						
Population	Total No. animals	SNP (Allele frequency)	LSM ± SE (Genotypes, No. animals)			*P*-value
M × P (F_2)	589	c.236+142A>G (A, 0.70)	40.28 ± 0.32 (AA, 275)	41.56 ± 0.31 (AG, 269)	41.64 ± 0.77 (GG, 45)	0.014
	589	c.630C>T (C, 0.56)	40.05 ± 0.40 (CC, 177)	41.38 ± 0.29 (CT, 300)	41.32 ± 0.48 (TT, 112)	0.018
Piétrain	865	c.236+142A>G (A, 0.46)	64.80 ± 0.40 (AA, 190)	65.54 ± 0.27 (AG, 419)	64.96 ± 0.34 (GG, 256)	0.21
	1173	c.630C>T (C, 0.38)	65.01 ± 0.41 (CC, 185)	65.52 ± 0.24 (CT, 532)	64.78 ± 0.25 (TT, 456)	0.10
German Landrace	536	c.236+142A>G (A, 0.92)	44.92 ± 0.24 (AA, 456)	45.89 ± 0.58 (AG, 79)	42.35 ± 5.08 (GG, 1)	0.26

Haplotype analysis						
Population	Total No. animals	c.236+142A>G - c.630C>T (Haplotype frequency)	LSM ± SE (Haplotype copy number, No. animals)			*P*-value
			(0)	(I)	(II)	
M × P (F_2)	589	HT1_A-C (0.56)	41.32 ± 0.48 (112)	41.38 ± 0.29 (300)	40.05 ± 0.40 (177)	0.018
	589	HT2_A-T (0.14)	41.04 ± 0.26 (429)	40.98 ± 0.44 (155)	40.28 ± 2.62 (5)	0.97
	589	HT3_G-T (0.30)	40.28 ± 0.32 (275)	41.56 ± 0.31 (269)	41.64 ± 0.77 (45)	0.014
Piétrain	849	HT1_A-C (0.35)	65.01 ± 0.28 (378)	65.68 ± 0.29 (356)	64.40 ± 0.51 (115)	0.054
	849	HT2_A-T (0.12)	65.21 ± 0.21 (664)	65.17 ± 0.41 (173)	65.62 ± 1.56 (12)	0.96
	849	HT3_G-T (0.50)	64.86 ± 0.38 (209)	65.64 ± 0.26 (429)	64.67 ± 0.37 (211)	0.059
	849	HT4_G-C (0.04)	65.14 ± 0.19 (787)	66.13 ± 0.71 (60)	65.57 ± 5.39 (2)	0.41

In an attempt to extend the result obtained in the F_2 population, association between *PRKAA2* genotypes and traits of interest was studied in two commercial pig populations. No significant association with LMA was found for the two analyzed SNPs in the Piétrain population and for the segregating c.236+142A>G SNP in the German Landrace population (Table 2.4). Nevertheless, it should be noted that there could be limited power for the association study in the German Landrace population due to the low frequencey of the c.236+142A>G SNP. The haplotype analysis revealed that both HT1 (A-C) and HT3 (G-T) showed a tendency towards association with LMA in the Piétrain population. Animals carrying two copies of HT1 (A-C) seemed to have less LMA compared to the other two types of animals in both the M × P F_2 and Piétrain populations (Table 2.4). HT3 (G-T) in the M × P F_2 population tended to increase LMA, whereas animals with two copies of HT3 (G-T) showed the smallest LMA among the three different types of animals in the Piétrain population.

Considering the versatile biological functions of α2 AMPK, *PRKAA2* was investigated as a functional candidate gene for muscle growth and fat deposition in pigs. Interestingly, our mapping analysis assigned *PRKAA2* to a region on SSC6 where QTL for loin muscle area, fatness and IMF were reported (Ovilo *et al.* 2002b; Mohrmann *et al.* 2006; Edwards *et al.* 2008). We detected significant associations of the *PRKAA2* genotypes with LMA in the M × P F_2 population. However, a classical QTL segregation analysis based on six microsatellite markers on SSC6 and the *PRKAA2* polymorphism in 167 M × P F_2 animals (i.e. assuming fixation of alternative QTL alleles in M and P) (Haley *et al.* 1994) did not detect a QTL on SSC6 affecting LMA after excluding the interference of the *RYR1* mutation (data not shown). The inability to detect the reported QTL in the *PRKAA2* region in our pig population might be due to the small number of animals used in the analysis and the assumption of alternatively fixed alleles, which does not apply to the *PRKAA2* polymorphisms. Nevertheless, given a large number of the animals (n = 1173) used and high polymorphism level of the two associated SNPs and haplotypes HT1 (A-C) and HT3 (G-T) in the Piétrain population (Table 2.4), we would have a high probability of detecting the same associations in this population too. However, we only observed suggestive associations in the Piétrain pigs.

Not much work has been carried out to analyze the candidate genes situated in the region on SSC6 where the QTL affecting LMA was reported (Ovilo *et al*. 2002; Mohrmann *et al*. 2006; Edwards *et al*. 2008). The leptin receptor gene (*LEPR*) has been analyzed as a very interesting candidate gene for this reported QTL. However, no significant associations were found between sequence variants in porcine *LEPR* and LMA (Ovilo *et al.* 2005). In the present study, only weak associations between the *PRKAA2* genotypes and LMA were found.

In summary, we presented the genomic characterization of the porcine *PRKAA2* gene and provided an initial evaluation of association between this gene with lean- and fat deposition in a large sample of pigs. Weak associations were found between the *PRKAA2* genotypes and muscle development in the investigated populations.

Chapter 3

Genomic characterization and polymorphism analysis of genes relevant to lipid metabolism in pigs

Abstract

Fatness, intramuscular fat content (IMF) and fatty acid composition are important pork quality traits. We hypothesized that genes encoding key enzymes or key regulators in lipid metabolism might affect these lipid-related traits. Hence, eleven such genes (*ACACA*, *ACACB*, *CPT1A*, *CPT1B*, *CPT2*, *MLYCD*, *FADS1*, *FADS2*, *FADS3*, *SCD1* and *SCD5*) were analyzed here. Ten of these genes have not been previously characterized in pigs. Their genomic structures were elucidated by shotgun sequencing of porcine BACs and semi-automatic annotation. A total of 367 sequence variants were identified in the eleven genes by re-sequencing approximately 85.5-kb in twelve parental animals of a Mangalitsa × Piétrain cross. Nine variants found in six genes (*ACACA*, *ACACB*, *CPT2*, *MLYCD*, *FADS2* and *SCD1*) were further investigated in 580 pigs of the Mangalitsa × Piétrain F_2 population. Four of them showed associations with the lipid-related traits at a nominal $P \leq 0.05$. An association of n-3 polyunsaturated fatty acid (n-3 PUFA) content in *longissimus dorsi* muscle was found for an intronic variant of 280-bp insertion/deletion in *ACACA*. A promoter SNP and a non-synonymous SNP in *ACACB* were associated with the n-3 PUFA content. The non-synonymous variant in *ACACB* also showed an association with backfat thickness at mid-back. An intronic SNP in *FADS2* showed an association with IMF and several backfat thickness measurements. Finally, allelic frequencies of the nine variants were determined in 176 pigs belonging to the Piétrian, German Large White, German Landrace and Duroc populations.

Keywords association, gene structure, lipid metabolism, polymorphism, pork quality

Introduction

Fatness and intramuscular fat content (IMF) are important pork quality traits because consumers desire both leanness and palatability in pork. Moreover, pork fatty acid composition is considered another crucial aspect of quality due to its relevance to human health. For example, excessive intake of saturated fatty acids (SFA), particularly myristic acids and palmitic acids, is often associated with a high risk of cardiovascular diseases (Williams 2000), while increased intake of monounsaturated and polyunsaturated fatty acids (MUFA and PUFA, respectively) is favorable due to their cholesterol decreasing effect (Stewart *et al*. 2001; Lichtenstein 2006).

Backfat and intramuscular fat are the consequences of lipid deposition in adipose tissue and muscle respectively, indicating that lipid metabolism plays an important role in their development. Different lipid metabolic pathways, therefore, are also involved in the determination of fatty acid composition in backfat and intramuscular fat. Thus, it is logical to analyze candidate genes relevant to lipid metabolism for these lipid-related traits in pigs. Obviously, genes encoding key enzymes or key regulators in different pathways of lipid metabolism are the prime choices.

In this study, eleven candidate genes relevant to fatty acid biosynthesis (*ACACA*), oxidation (*ACACB*, *CPT1A*, *CPT1B*, *CPT2* and *MLYCD*) and desaturation (*FADS1*, *FADS2*, *FADS3*, *SCD1* and *SCD5*) were investigated. There are alpha and beta acetyl-CoA carboxylase isoforms (ACACA and ACACB, respectively) in mammals (Barber *et a*l. 2005). ACACA catalyzes the first and committed step in *de novo* fatty acid biosynthesis, whereas ACACB regulates fatty acid oxidation in mitochondria via its product, malonyl-CoA. Malonyl-CoA decarboxylase (MLYCD) that catalyzes the breakdown of malonyl-CoA is also thought to be a key regulator of fatty acid oxidation (Sacksteder *et al*. 1999; Lee *et al*. 2004). The transport of long-chain fatty acids into mitochondria for oxidation requires at least two key enzymes: carnitine palmitoyltransferase I and II (van der Leij *et al*. 2000). At least two CPT I isoforms (live type, CPT1A and muscle type, CPT1B) and one ubiquitous CPT II (CPT2) have been identified in mammals. CPT I is tightly regulated by its physiological inhibitor malonyl-CoA. Hence, it explains that ACACB and MLYCD, responsible for the formation and turnover of cellular malonyl-CoA respectively, are involved in the regulation of fatty acid oxidation. In mammals, Δ5, Δ6 and Δ9 desaturases are responsible for

synthesis of most of the unsaturated fatty acids. The Δ9 desaturase, also called stearoyl-CoA desaturase (SCD), is the key enzyme in the biosynthesis of MUFAs such as palmitoleic acids and oleic acids (Enoch *et al*. 1976). Two SCD isoforms (SCD1 and SCD5) were found in humans (Wang *et al*. 2005) and cattle (Lengi & Corl, 2007), and four (Scd1 through Scd4) in mice (Miyazaki *et al*. 2003). The porcine SCD1 gene (*SCD1*) has been previously characterized (Ren *et al*. 2004b). Our work provides evidence for the existence of porcine *SCD5*. Due to the absence of Δ 12 and Δ 15 desaturases in mammals, linoleic acids (n-6) and alpha-linolenic acids (n-3) must be provided in the diet. However, using the two essential fatty acids as starting points, longer and more complex n-3 and n-6 PUFA can be formed through a combination of elongation and desaturation reactions in mammals (Nakamura & Nara 2004). The Δ5 and Δ6 desaturases, encoded by *FADS1* and *FADS2* respectively, are the key enzymes in the desaturation reactions. In humans, *FADS3* encoding fatty acid desaturase 3 is clustered with *FADS1* and *FADS2*, and also shows significant homology with them (Marquardt *et al*. 2000).

The objectives of this study were to elucidate genomic structures of candidate genes, to detect sequence variants and to evaluate association between the identified variants and the lipid-related traits in 580 pigs of a Mangalitsa × Piétrain F_2 population.

Materials and methods

Animals and phenotypes

A three-generation resource family of a cross between the Mangalitsa and Piétrain pig breeds (M × P) was used in the study. Two Mangalitsa boars, homozygous for the *RYR1* normal c.1843C allele, were mated to 13 Piétrain sows homozygous for the mutation (c.1843T, Fujii *et al*. 1991). Five males and 18 females of their offspring were used to produce 613 F_2 animals. Animals were fed *ad libitum*. F_2 males were castrated. All the F_2 pigs were slaughtered at body weight of 90-100 kg. The lipid-related traits were recorded including IMF, fatty acid composition and fatness (Table 3.1). IMF in a sample of the *longissimus dorsi* muscle at rib 12th-13th was measured using the lipid extraction method suggested by Bligh & Dyer (1959) and modified by Hallermayer (1976). Fatty acid composition of intramuscular fat was determined by gas chromatography and expressed in % total fatty acids. Backfat thickness measurements (BFT) were obtained according to the German performance test directives (ZDS 2004).

A panel of unrelated animals of four breeds was used for allele frequency estimation, comprising: Piétrian (PI, n = 45), Duroc (DU, n = 39), German Large White (DE, n = 49) and German Landrace (DL, n = 43).

Table 3.1 Means and standard deviations (SD) of twelve phenotypes measuring fat deposition and muscle fatty acid profile in the F_2 population (n = 613).

Description	Trait	Mean	SD
Fat deposition			
Backfat thickness at shoulder [mm]	BFTW	45.35	6.19
Backfat thickness at mid-back [mm]	BFTM	29.35	5.48
Backfat thickness at loin [mm]	BFTL	26.59	6.2
Side fat thickness [mm]	BFTS	42.96	8.78
Average backfat thickness [mm]	ABFT[1]	33.76	5.08
Intramuscular fat content [%]	IMF	2.06	0.63
Fatty acid content in IMF			
Saturated fatty acids [%]	SFA[2]	36.66	2.02
Monounsaturated fatty acids [%]	MUFA[3]	47.34	3.46
Polyunsaturated fatty acids [%]	PUFA[4]	14.88	4.03
n-3 polyunsaturated fatty acids [%]	n-3PUFA[5]	1.27	0.43
n-6 polyunsaturated fatty acids [%]	n-6PUFA[6]	13.61	3.84
Polyunsaturated to saturated ratio	P/S[7]	0.41	0.12

[1]Calcalated as the average of three measurements (BFTW, BFTM and BFTL)

[2]SFA = C14:0 (Myristic acid) + C16:0 (Palmitic acid) + C18 :0 (Stearic acid)

[3]MUFA = C16:1 (Palmitoleic acid) + C18:1 (Oleic acid)

[4]PUFA = n-3PUFA + n-6PUFA

[5]n-3PUFA = C18:3 (Linolenic acid) + C20:3 (Eicosatrienoic acid) + C20:5 (Eicosapentaenoic acid) + C22:6 (Docosahexaenoic acid)

[6]n-6PUFA = C18:2 (Linoleic acid) + γ_C18:3 (Gamma-linolenic acid) + C20:4 (Arachidonic acid) + C22:4 (Docosatetraenoic acid)

[7]P/S = PUFA/SFA

In silico porcine BAC library screening and BAC sequencing

Over 600,000 BAC end sequences (BES) have been generated from four porcine BAC libraries: CHORI-242 (http://bacpac.chori.org/porcine242.htm), RPCI-44 (Fahrenkrug *et al*. 2001), PigE (Anderson *et al*. 2000) and INRA-PigI (Rogel-Gaillard *et al*. 1999). BLAST results of non-repetitive BES against the human reference sequence were available to search by human location through the website (http://www.sanger.ac.uk/cgi-bin/Projects/S_scrofa/BESsearch.cgi).

It was assumed that if BES hits of some clone were anchored to the human location containing a gene of interest, this clone probably contained the porcine corresponding gene. Therefore, it was possible to identify porcine BACs for the selected genes based on the available BES information. Subsequent colony PCRs using porcine specific primers (Table 3.2) were performed for further confirmation. Positive BACs were sequenced using a shotgun approach described in Retter *et al*. (2007). Data were assembled and edited using the GAP4 program (Staden 1996).

Semi-automatic gene annotation

Contigs containing genes of interest were identified by comparing human mRNA sequences of the genes with all contigs derived from BAC sequencing using BLAST analysis with an E-value threshold of 1e-10 through a local server. The *GenomeThreader* software (Gremme *et al*. 2005) was used to predict the genomic structures of the genes based on the identified contigs and the Dana-Farber Cancer Institute (DFCI) gene indices (Quackenbush *et al*. 2001). The *GenomeThreader* output was viewed and edited using the Apollo sequence annotation editor (Lewis *et al*. 2002).

Table 3.2 Primers used for colony PCRs.

Gene / primer number	Primer sequence (5'-3')	Primer binding region	Product size [bp]	Annealing temperature
ACACA_4483up	CCGTGCAGTTTGCTGACTTG	Exon 53		
ACACA_4484dn	AGCTCGGGGTTGGCATTGT	Exon 54	832	58°C
ACACA_4485up	CTCTAGTGAAAATCCAGATGAGG	Exon 13		
ACACA_4486dn	GGCTTGTGAACCAATGTCCA	Intron 13	447	58°C
ACACA_4487up	ACAACTCAGCTTGGCTTGCT	Intron 30		
ACACA_4488dn	CTGACACGGTGGAGTGAATG	Exon 31	502	60°C
ACACB_4489up	CTGGAGGACCAGGTTAAGCA	Exon 55		
ACACB_4490dn	TGATGTTCTCTCGGATGGTG	Exon 56	316	60°C
ACACB_4491up	TCATGCCTCTCTGTGTTTGC	Intron 7		
ACACB_4492up	TGAACATGGCTGGTATCTCG	Intron 8	376	60°C
CPT1A_4471up	CAATGTGCTTTCTGGTCAGG	Intron 15		
CPT1A_4472dn	CGCCCGCTATCTTGAATAAC	Exon 16	362	58°C
CPT1A_4473up	TCTCTCCTTTTCGCCAGTGT	Exon 14		
CPT1A_4474dn	CCTCGGGTCTCACCTTGTAA	Intron 14	195	60°C
CPT1B_4475up	AACAGCGGGTTCCTCCTACT	Exon 3		
CPT1B_4476dn	GGAAAGCAGCAGTTTCAAGG	Exon 4	485	60°C
CPT1B_4477up	AGCCTCGATGACTCGAATGT	Exon 15		
CPT1B_4478dn	CCACGTAAAGGCAGAAGAGG	Exon 16	332	60°C
CPT2_4479up	TGACCGACACTTGTTTGCTC	Exon 5		
CPT2_4480dn	TGCAGCCTATCCAGTTGTTG	Exon 5	215	60°C
CPT2_4481up	CTCGAAACCCCATTGTCTTG	Exon 4		
CPT2_4482dn	GGGAGTCGAGTGGAATTGAA	Exon 4	313	60°C
MLYCD_4499up	GAGCGAACTGTTCACCGATG	Exon 5		
MLYCD_4500dn	CCACAGGGTTGAGCGAGTAG	Exon 5	206	58°C
MLYCD_4513up	CGCGGACTTTATGAGCTTCT	Exon 1		
MLYCD_4514dn	AGCTTGCTGATGTGATGGAA	Exon 1	237	58°C
FADS1_3813up	TTGCGACACCTGTCCGTCTT	Exon 4		
FADS1_3814dn	ATGTTGATGTCTGCGTCTTTGC	Exon 5	255	60°C
FADS1_3850up	GGCTCACTTATGTGCCACTGTT	Exon 8		
FADS1_3851dn	GGACTCGTACTCTATACCATGCTTG	Exon 11	894	60°C
FADS2_3815up	TGATTGACTGCGAGCCCTAC	Exon 9		
FADS2_3816dn	CATTGAAAAACGACTACTCCACG	Exon 10	409	57°C
FADS3_4451up	TCCTCGCCTGGCTTATCATC	Exon 3		
FADS3_4452dn	GGTCACGTCTGGGTCCTTGT	Exon 5	844	60°C
FADS3_4453up	GTGGAGCCCTCCCTCTTCAT	Exon 10		
FADS3_4454dn	CCCTGCGGTAGTTGTGCCTT	Exon 11	234	60°C
SCD5_3817up	ATGCTTCCTCCTAAACCGCT	Exon 2		
SCD5_3818dn	GCGACTGCAAGAAATATCCTCA	Exon 2	106	56°C

Sequence variant identification

Screening for sequence variants was performed by re-sequencing twelve parental animals of the M × P family containing two Mangalitsa and ten Piétrain pigs. Primers for both PCR amplification and sequencing (Table 3.3) were selected from the annotated BAC sequences. Sequencing reactions were carried out with the BigDye Terminator v1.1 Cycle Sequencing Kit and were resolved using ABI 377 DNA sequencer (Applied Biosystems). Base calling, sequence assembly and variant detection were conducted using the Phred/Phrap/Polyphred/Consed software (Nickerson *et al*. 1997; Ewing & Green 1998; Ewing *et al*. 1998; Gordon *et al*. 1998).

Table 3.3 Primers used in PCR- re-sequencing for sequence variant detection.

Gene/Primer number	Primer sequences (5'-3')	Primer binding region	Product size (bp)	Annealing temperature
ACACA				
ACACA_5303up	GAGCAGTTAGCCAGACTTTTGA	Exon 1		
ACACA_5304dn	ATTTCGACGTTCCAGAAGCA	Exon 1	345	60°C
ACACA_5912up	TCTTGCAGTGCTACTCATGGAT	Exon 1		
ACACA_5913dn	TGGGACATACCTAGCCCTCA	Intron 1	211	59°C
ACACA_5305up	TTTTGAGTGTGTAATGCTTTTGG	Intron 3		
ACACA_5306dn	GCCTATATTCTTCTGTGCAAGGT	Intron 4	378	60°C
ACACA_5189up	TGCCATTGGAAGTGCTGTTA	Intron 5		
ACACA_5190dn	TGCTAAGGAGGCAGAAAGGA	Intron 6	390	60°C
ACACA_4876up	TTGAAACACTGGCTTGTCAGA	Intron 6		
ACACA_4877dn	ATGGCATTCTCAAACTGATGTT	Intron 7	698	57°C
ACACA_5200up	ATCCCATGAATGCAGTTTGT	Intron 7		
ACACA_5201dn	GGCTACGCCCTTGTGTGTAA	Intron 8	296	57°C
ACACA_4880up	TTGGAGTAGGGCAGGTCTTG	Intron 8		
ACACA_4881dn	GGGAGTAAGGACCAAAATGACA	Intron 9	521	57°C
ACACA_5898up	TGACAGAAAGGAGGCTAAAATG	Intron 9		
ACACA_5899dn	ACCCAAGCCATAATCCCAAA	Intron 10	231	60°C
ACACA_5202up	GCAGAAAATTGACGAGAACTGA	Intron 10		
ACACA_5203dn	TCCAGGTGAGCCTATTGCTT	Intron 11	384	57°C
ACACA_5900up	TGCCAGTCTTCTCCTTCACTAC	Intron 11		
ACACA_5901dn	AGACATCCAAATGATAGCACGA	Intron 12	240	60°C
ACACA_5204up	TTGGCTGTTTGGAACTGATG	Intron 12		
ACACA_5205dn	AAGTGTCTGCTGGCAACTGTA	Intron 13	720	60°C
ACACA_5206up	CAGTGTTCACCCAGCTTTGA	Intron 13		
ACACA_5207dn	CCAGTTTTACAGGCACCTCTG	Intron 14	456	60°C
ACACA_5902up	CTGAGAAAAACGAGTGATGTGTG	Intron 14		
ACACA_5903dn	GAGAGGGGGTGGGATGTAA	Intron 15	241	60°C
ACACA_4485up	CTCTAGTGAAAATCCAGATGAGG	Exon 15		
ACACA_4486dn	GGCTTGTGAACCAATGTCCA	Intron 15	447	58°C
ACACA_5208up	ATAGGCTTCATCAGGGCAGA	Intron 15		

ACACA_5209dn	TGGATCAGAGCAGCAAACAG	Intron 16	617	60°C
ACACA_5210up	CTCCCTCCTAAGATGCCAAA	Intron 16		
ACACA_5211dn	TGCCTCCCTTCAAATAGACC	Intron 17	472	60°C
ACACA_5212up	TTGAGCAACAGGATGGAGAG	Intron 17		
ACACA_5213dn	ACCTAGAGCCCCCAAATCAG	Intron 18	422	60°C
ACACA_5214up	CCAGGAAATGCTGTTACCAA	Intron 18		
ACACA_5215dn	GAGGCGAGAAGCGAGAAGTA	Intron 19	227	60°C
ACACA_5216up	GACTTCATCTGGGAGGGACA	Intron 19		
ACACA_5217dn	TTGACTGATGGGAGGAAAGG	Intron 20	264	60°C
ACACA_5218up	AACGTACATACTCCCACTTATTTCC	Intron 20		
ACACA_5219dn	TGTCCCTGTCAACCCTTTTC	Intron 21	243	60°C
ACACA_5220up	TGAACATGAGACACGGGAGA	Intron 21		
ACACA_5221dn	ATTCCTCGTGCAAAATCAGG	Intron 22	492	60°C
ACACA_5222up	TTGGCATCACGGAACTAGAG	Intron 22		
ACACA_5223dn	CAGTGACACAGATAAGAAGCAAGG	Intron 23	533	60°C
ACACA_5375up	TAAATCTGGGCTCTGGGTGT	Intron 25		
ACACA_5376dn	GACTGGGGAAAGGAGGAAAG	Intron 26	305	58°C
ACACA_5377up	GCGAGCACCAGTTCTTCTTG	Intron 26		
ACACA_5378dn	CCAAACCCCACTCATCTCAC	Intron 27	279	58°C
ACACA_4487up	ACAACTCAGCTTGGCTTGCT	Intron 32		
ACACA_4488dn	CTGACACGGTGGAGTGAATG	Exon 33	502	60°C
ACACA_5161up	GCATCAAATGGTCCCTGACT	Intron 40		
ACACA_5162dn	CAGCCCCTCTAGGTCAAATG	Intron 41	518	60°C
ACACA_4962up	TGTCTCTGACATGGGCTCAC	Intron 44		
ACACA_4963dn	CTTCATCCTCCACATGCTCA	Exon 45	1258	60.6°C
ACACA_5904up	ATGTTCTCGTTTCCCACAGA	Intron 43		
ACACA_5905dn	AAATCTCTACCTCTCTCCCACCT	Intron 44	504	60°C
ACACA_4923up	TTGGAACAAGCTGCATTACG	Intron 44		
ACACA_4924dn	TCTCTTGGTAGGACTGTAGCTGA	Intron 45	466	60°C
ACACA_5307up	CCTCCTGGTAATTGCTGCTC	Intron 45		
ACACA_5308dn	GCCACACAGTCAGCTCTTCA	Intron 46	249	60°C
ACACA_5906up	TCTGTGCCTTAGTTCTTATACCTTG	Intron 46		
ACACA_5907dn	TCACATACTTTCTCCCAAATGCT	Intron 47	216	60°C
ACACA_5309up	AGCTCTGTGTCAGGGAGGAA	Intron 47		
ACACA_5310dn	GTCCTATGGGCTCTGTCGTG	Intron 48	288	60°C
ACACA_5311up	TAACGATTGATGCTGGGTTG	Intron 48		
ACACA_5312dn	GAAAGGAAGAATGAAAGCTGGA	Intron 49	248	60°C
ACACA_5908up	GAAATAGGAGATGAGGTCTGAAGG	Intron 49		
ACACA_5909dn	GGGTAGAGAGGGTGACTGGTG	Intron 50	640	60°C
ACACA_5313up	CGTGTCTTGATGTGGGATTG	Intron 50		
ACACA_5314dn	CTCTAAGGCTGGTGGTCTGC	Intron 51	283	60°C
ACACA_5315up	TGGAATCAAGACAAGGAGTGA	Intron 51		
ACACA_5316dn	CAGAAAAGCGGACCAGAGAC	Intron 52	665	60°C
ACACA_5317up	TGCTCCCTGGTTTCTGATGT	Intron 52		
ACACA_5318dn	TCCAGCATGATTCCCTTCTC	Intron 53	395	60°C
ACACA_5910up	CTCGTTCCGTTGTCTCCTTC	Intron 53		
ACACA_5911dn	CGGGTGACTTCTGCTTTCTT	Intron 54	400	60°C
ACACA_5319up	TGGATGTGGGAAAAGAGGAG	Intron 54		
ACACA_5320dn	CATGCAAGAGGCAGACACAG	Intron 55	631	60°C

ACACA_5163up	CTGAACTGGGGCAACTAAGC	Intron 55		
ACACA_5164dn	GTGTTACTGTCGGGGAGGAA	Intron 56	363	60°C
ACACA_5321up	AGCATTTCCCTCTCAACCAG	Intron 56		
ACACA_5322dn	GCCTCACCCTTTGTTCCTAA	Intron 57	565	60°C
ACACA_5323up	CATGACCCAGCACATCTCAC	Exon 58		
ACACA_5324dn	CTTGCAGATTTCACGTTCCA	3' end	665	60°C
ACACB				
ACACB_5167up	GTGGCTCTCCCAGAAAACAA	5' flanking		
ACACB_5168dn	AGCTTGTAGGGCAAAGGTCA	5' flanking	525	60°C
ACACB_5088up	ATTCAGGCATGGACTTGGAC	5' flanking		
ACACB_5034dn	GATGAACTGACCCCTGCTGT	Exon 1a	832	60°C
ACACB_5035up	TCCAGGAGAGCCAAAATGAC	5' flanking		
ACACB_5036dn	CATTCTGTTTGGAGCCCTTC	Intron 1a	501	60°C
ACACB_5037up	AACTGTGAATTGGGGGAGTG	5' flanking		
ACACB_5038dn	AGCCAGAGGCTGGTATGATG	Exon 1b	511	60°C
ACACB_5039up	CTTTTGCAGAGGGCTACAGG	5' flanking		
ACACB_5040dn	GGTCATAACGAAGGCAGGAA	Intron 1b	537	60°C
ACACB_5041up	GGCTCTCACATCAGCTCCTT	Intron 1		
ACACB_5042dn	TTTCACCTGGCTACCCTCAC	Intron 2	849	60°C
ACACB_5043up	TTCGGCTTCTCTGTCCATGT	Intron 2		
ACACB_5044dn	GGTCACACACATGTTGCTTCA	Intron 3	344	60°C
ACACB_5072up	CAGCCTTCAGCACAGGTAGG	Intron 3		
ACACB_5073dn	AGGGTAGAGGAGATGAGAGTAGG	Intron 4	250	60°C
ACACB_5074up	CCCTCAACTTCTGACCCTCA	Intron 4		
ACACB_5075dn	TCAAGCTGGTCCCTCCATAC	Intron 6	794	60°C
ACACB_5076up	AGCTGGGACCTGGGTACTTT	Intron 6		
ACACB_5077dn	CTCGGGTCACTCCTCACTTC	Intron 7	284	60°C
ACACB_5078up	TTGTGTCTGGGCTGTTCTTG	Intron 7		
ACACB_5079dn	AATGCCCTCTTGATGGTGAC	Intron 9	492	60°C
ACACB_5080up	CGTGCCTGCTTTGGATAACT	Intron 9		
ACACB_5081dn	GGGCAGGGAAGTCAGATTAG	Intron 11	799	60°C
ACACB_5082up	GCACAGGATGTTGTTTTGTGA	Intron 11		
ACACB_5083dn	CTCGTCCAGGTTAAGGTTCG	Intron 12	492	60°C
ACACB_5084up	ACACCTGCACCCCTAGTGAG	Intron 12		
ACACB_5085dn	CCTCCCTTCATCTCAAGTGC	Intron 13	414	60°C
ACACB_5086up	TGGGAAAACAACCTGTGTCC	Intron 13		
ACACB_5087dn	CCTGCTTCTGCCTTAACTGC	Intron 15	442	60°C
ACACB_5111up	AGCAAGAGCGGATGTCCTTA	Intron 15		
ACACB_5112dn	AGCATGAGAAAACCCACCAC	Intron 16	404	60°C
ACACB_5113up	CGAGGGCTAGGACTCAACAG	Intron 16		
ACACB_5114dn	CTTGCTCTGATGGGTGTGAA	Intron 17	598	60°C
ACACB_5115up	AAGGCTGTCAGTGGGTCAGT	Intron 17		
ACACB_5116dn	GTGCAGCAGCATTGTCAGAT	Intron 18	531	60°C
ACACB_5117up	CAAACCCTCTGCTGGAACTC	Intron 18		
ACACB_5118dn	TGGGAGCCTCAAGTCTGAAC	Intron 19	340	60°C
ACACB_5169up	CAGGAAGTGGAGCTGGTCAT	Intron 22		
ACACB_5170dn	GCTCTGGGTACAGGGGATCT	Intron 24	384	60°C
ACACB_5129up	GGGCTTGTGTATCAGGCTGT	Intron 24		
ACACB_5130dn	GAGGAAGCAGTTCTGGGATG	Intron 26	841	60°C

ACACB_5171up	AGAGCGAACACTGCAAGGTG	Exon 25		
ACACB_5172dn	GTGGGAGGCGATCAGGAC	Exon 26	196	60°C
ACACB_5131up	TTGGGAAGGCTCTAATGTGG	Intron 28		
ACACB_5132dn	GCTCAGGGTGTTCTGGAGAG	Intron 29	289	60°C
ACACB_5173up	GCACCCACAGGAACTTTGAT	Intron 29/Exon 30		
ACACB_5174dn	CACATGGACACCTCACCAAG	Intron 31	371	60°C
ACACB_5133up	ACTCCCCATGTGTCCGTATG	Intron 33		
ACACB_5134dn	CTAAACCCTGAGTGGCAGGA	Intron 34	533	60°C
ACACB_5135up	CTGAAATTGAATCGCTGTGG	Intron 35		
ACACB_5136dn	GGGAATGGAGAACTGGGATT	Intron 36	404	60°C
ACACB_5175up	CTGAGACTTGCCTCCCTCAC	Intron 36		
ACACB_5176dn	GCATTCAAAGGACCAGAAGG	Intron 37	529	60°C
ACACB_5121up	CAACAACAGAAGCGAGGTGA	Intron 38		
ACACB_5122dn	TACAGCAGGAGCTCAGTGGA	Intron 39	439	60°C
ACACB_5123up	CCCTGCCCACAAAGTAGTGT	Intron 39		
ACACB_5124dn	ATGAACCACCAGCAAAAAGG	Intron 40	259	60°C
ACACB_5125up	GGCTGCTTTATCTGCTTTGG	Intron 40		
ACACB_5126dn	GGAGCCAGGGATGGTACATA	Intron 41	359	60°C
ACACB_5127up	CTCCCTGGATTACGACGAGA	Exon 41		
ACACB_5128dn	CTACAGGTCCACTCCCCAGA	Intron 42	631	60°C
ACACB_5137up	CAGGACCTTGCTAACCATGC	Intron 42		
ACACB_5138dn	TAAACCCCAGCCACATTTTC	Intron 43	290	60°C
ACACB_5139up	TGAAGTGTCAGACCCAGTGC	Intron 43		
ACACB_5140dn	CACGCTCCTTTATCCCAGAG	Intron 44	387	60°C
ACACB_5177up	TGGCAACCACAAGTCTGTTC	Intron 44		
ACACB_5178dn	CTAAGGCCACAAGCAAATCC	Intron 45	373	60°C
ACACB_5179up	CCCGACTGAAAACGGTACAC	Intron 45		
ACACB_5180dn	AGAACCCACGAAAACAGCAG	Intron 46	294	60°C
ACACB_5181up	TATTGATGCCAATGCAGAGC	Intron 46		
ACACB_5182dn	TGATGATGGGACCATTCAGA	Intron 47	326	60°C
ACACB_5183up	CTCCCACCTGACCACACTCT	Intron 47		
ACACB_5184dn	CCCTTTGGAAGCTCAGAACA	Intron 48	416	60°C
ACACB_5119up	ACTGCATGAGCGACTTGATG	Intron 49		
ACACB_5120dn	TGTGTGTGTTGACTGCAGGA	Intron 50	289	60°C
ACACB_5187up	ACCCTGCCCTATTGCTCTGT	Intron 50		
ACACB_5188dn	CACCGTCTGGTTGTTGTCC	Exon 52	650	60°C
ACACB_5143up	CTTGCGGTTGTTCTCATTCC	Intron 52		
ACACB_5144dn	CGCTTAAGTCAAGAGCTGGTC	3' flanking	706	60°C
CPT1A				
CPT1A_5846up	CGTGTCTGTCGGTAGAATGG	Intron 2		
CPT1A_5847dn	GGAATGCAGAACAGCAACAG	Intron 3	256	60°C
CPT1A_5244up	GGGTCGTCCTGTGTCTCATC	Intron 3		
CPT1A_5245dn	CAGGTGTGTGTCTGCAAAGG	Intron 4	444	60°C
CPT1A_5848up	TCTCACCTTTTTGCGTGTTG	Intron 4		
CPT1A_5849dn	TGCCAGTAAATGAATGTCTGCT	Intron 5	245	60°C
CPT1A_5246up	GTACCTGGAATCCGTGAAGC	Exon 6		
CPT1A_5247dn	CTGCTCCTCAAAACCTCAGC	Intron 6	291	60°C
CPT1A_5248up	TGTAACTCAGCACGAAGTGAAAA	Intron 6		
CPT1A_5249dn	GGAAAATAGAGGAGGAGAAGGTG	Intron 7	434	57°C

CPT1A_5850up	AACCAGTCTCTTTCCCAGCA	Intron 7		
CPT1A_5851dn	AAAAAGCAGTTTGTTTGCTTACC	Intron 8	257	60°C
CPT1A_5852up	GGGCTCCTTAAATTCGTGTG	Intron 8		
CPT1A_5853dn	ATCGGCGTGGTCTCCTCT	Intron 9	474	60°C
CPT1A_5250up	GGCTGCTGTATCGACTCCTC	Intron 9		
CPT1A_5251dn	AAGCCTGCCTCCTTCCTCT	Intron 10	574	60°C
CPT1A_5252up	CACTGCGGAGAAAATCACAA	Intron 10		
CPT1A_5253dn	AAGCCAGAGCTGACATCCAC	Intron 11	797	60°C
CPT1A_5254up	TGTCAGGAACAGGGGAGATG	Intron 11		
CPT1A_5255dn	CCGTAGGAAACAAAGGCAGA	Intron 12	442	57°C
CPT1A_5256up	CTTTGGGAGGTCAGGTGTG	Intron 12		
CPT1A_5257dn	ACGCTTTCAATGGGAGAAGA	Intron 13	414	57°C
CPT1A_5854up	GAGGAGGCGTCTGTGCTT	Intron 13		
CPT1A_5855dn	GGCTTCTGCTGGACACACAT	Intron 14	334	60°C
CPT1A_5856up	AGCTAATGCCGTTTCCACCT	Intron 14		
CPT1A_5857dn	CCTTCTCATGCTTTCTGTCC	Intron 15	466	60°C
CPT1A_5858up	TCCTTTGCTTCTTAGGTGGA	Intron 15		
CPT1A_5859dn	TAGCCCGTGATTATTTTCGT	Intron 16	362	60°C
CPT1A_5258up	CTTCTCGGGGACGGAACA	Intron 16		
CPT1A_5259dn	GCATGAATAACCAGCAGGAG	Intron 17	303	60°C
CPT1A_5860up	TGAATGTGCTTTTCCGTAGGT	Intron 17		
CPT1A_5861dn	GTCTCCCATCAAATCAAAGAGA	Intron 18	203	60°C
CPT1A_5260up	GTGACAAGGCTGGAAAGGTG	Intron 18		
CPT1A_5261dn	GTCATTCACTCCACGGCAAG	Intron 19	481	60°C
CPT1B				
CHKB_4815up	AGCCGAGTGTTCTCACCAGT	Intron 10		
CHKB_4816dn	CACTTTCAAGCCCAGATCGT	Exon 11-3' UTR	454	60°C
CPT1B_5886up	TCTTCCTTCACACCCTGTCC	Promoter		
CPT1B_5887dn	GAATCTACCCGCCTCTCTGT	Promoter	500	60°C
CPT1B_5224up	TGGAGATCAGGGAACAGGAG	Promoter		
CPT1B_5225dn	CTCCAACCACAGGGAAAGG	Intron 1	529	60°C
CPT1B_5994up	GAGCAAAGCCCTTCTGTGAG	Exon 1		
CPT1B_5995dn	GGCTCTATTCTGCACCTGCT	Intron 2	572	56°C
CPT1B_5226up	TTGTCAAGGTCCTCCACTCC	Intron 2		
CPT1B_5227dn	CTCCAGCTCAGAACCCAAAG	Intron 4	945	60°C
CPT1B_5890up	CCTCCTTTGCTAGTTTCCATTC	Intron 4		
CPT1B_5891dn	GCCCACTCTTTCCTTATGTTC	Intron 5	250	60°C
CPT1B_5228up	CTGGACTCAGAGGGCTTGTC	Intron 5		
CPT1B_5229dn	TGTCAGCTATGGGGTCTCCT	Intron 7	672	60°C
CPT1B_5230up	CGCATTTCATCTCTGGCTCT	Intron 7		
CPT1B_5231dn	CCTGACCCTCTTTCAACGTC	Intron 9	495	60°C
CPT1B_5892up	GTCCACCTAGCCACCCAGA	Intron 9		
CPT1B_5893dn	CTGCTGCCGTTAGTTCCA	Intron 10	474	60°C
CPT1B_5234up	CAGCACCTCACAGACAGCA	Exon 10		
CPT1B_5235dn	TTAGTTCCAGCACCCCTCAC	Intron 11	367	60°C
CPT1B_5236up	TGAGGGGTGCTGGAACTAAC	Intron 10		
CPT1B_5237dn	GGACAGAGAGGAAGGCACAG	Intron 13	828	60°C
CPT1B_5238up	TCTGAGGGTTCGAGAGAGACA	Intron 13		

CPT1B_5239dn	GGCAGAAATGGAGCAGTGAA	Intron 15	716	60°C
CPT1B_5240up	GCTGCTCTTCACTGCTCCA	Intron 15		
CPT1B_5241dn	GCCCATCGACACTGACCT	Intron 16	201	60°C
CPT1B_5894up	TTGAGGAACAGCAGGAAGG	Intron 16		
CPT1B_5895dn	ACAAACCAGCCCACTTGAGA	Intron 17	583	60°C
CPT1B_5896up	GCAAGGGCTGGTTTACCTCT	Intron 17		
CPT1B_5897dn	AAGGGCATCTGGTGTTTCTC	Exon 19	362	60°C
CPT1B_4813up	CCACGTCTCCAGCAAGTTCT	Exon 18		
CPT1B_4814dn	CAGAAGCCTCCGCAAATAGT	Exon 19-3' UTR	600	55°C
CPT2				
CPT2_5345up	AGTCTCACATGGTCCCCTGA	Promoter		
CPT2_5346dn	TCTGCAACCTACACCACAGC	Promoter	502	60
CPT2_5343up	TGCTCAGTGGGTTAACAATCC	Promoter		
CPT2_5344dn	GAACCGCTTCGAGTCCTTC	Promoter	703	60
CPT2_4987up	GAGAAGGCCAAGGAAGGACT	Promoter		
CPT2_4988dn	CCCTTCTCCTCTTCCGAAAC	Promoter	587	60
CPT2_4983up	CCCAAGGACAGAGTCTGGAA	5' UTR		
CPT2_4984dn	GTACTGACCCCGCTGGAAG	Exon 1	550	60
CPT2_4985up	CTGCACACCAAAAACCACAC	Exon 1		
CPT2_4986dn	GGGTTCAGCAGAACAGTCGT	Intron 1	289	60
CPT2_4989up	TGGGCAGCCACTTAACTTCT	Intron 1		
CPT2_4990dn	CCTGGTAACCCAATCGAATC	Intron 2	259	60
CPT2_4991up	TGGAGACCCAGAGTTCCTTG	Intron 2		
CPT2_4992dn	CATTATGGAGGGCTCTGGAA	Intron 3	255	60
CPT2_4884up	CCCTGGTTTGACATGTACCT	Exon 4		
CPT2_4885dn	GCCTGGCTGCTGTCTTTAAA	Exon 4	839	60
CPT2_5001up	GCCGTGCTCAGGTTCTTAAA	Exon 4		
CPT2_5002dn	TATAGACAGGCTCCCCATGC	Intron 4	539	60
CPT2_4993up	TTCTGACTCCGGTTTTCCAC	Intron 4		
CPT2_4994dn	CACCTGAGGCATATGGAGGT	3’ end	741	60
FADS1				
FADS1_4558up	GAAGCGGGAGTTCATCAGGT	5' flanking		
FADS1_4561dn	CGCTCACCGTAGCATCCTG	Exon 1/ Intron 1	897	64°C (HotStart Taq + 5% DMSO)
FADS1_4852up	TTCTTCACCTGGGAGGAGGT	Exon 1		
FADS1_4853dn	GCTCAGCCCTTTGTTTGGTA	Intron 1	428	60°C
FADS1_4562up	TGCCTCACTGTTCTGCCTTCT	Intron 1		
FADS1_4563dn	GGAGATGCTGTGGGATCAAGT	Intron 2	564	60°C
FADS1_4564up	ACTCGGTGCCAAGCGTGATA	Intron 2		
FADS1_4565dn	ACCAAAGCTCCTGTGTTGTCCT	Intron 3	672	60°C
FADS1_4723up	TGCCTGTGTTCAGTGAGAGG	Intron 3		
FADS1_4724dn	CTGCATCCAAGCTCAGACAG	Intron 5	596	60°C
FADS1_4566up	CTGGCTCACTCTCCTCTGCT	Intron 5		
FADS1_4567dn	CCCATTCACACCCTTCTGTT	Intron 5	569	60°C (1x Q solution)
FADS1_4568up	TGGAGAAGAGCCAGGGTCAC	Intron 5		
FADS1_4569dn	GTTCCAGACGCAGCCTTAGC	Intron 6	256	60°C
FADS1_4854up	TTCCAGTGGTCTGAGGTGTG	Intron 6		
FADS1_4855dn	TCCCGTTCATCTTCTCCATC	Intron 7	399	60°C

FADS1_4570up	ACTGGAGGCAGAGAGACCTG	Intron 7		
FADS1_4571dn	CTCCCCCATCAGATCCTACC	Exon 8/Intron 8	280	60°C
FADS1_4572up	GGGTCCTAAATCTTGCCTCGT	Intron 9		
FADS1_4573dn	TGAGAAAGAACCTGGGCTGTG	Intron 11	638	60°C
FADS1_3850up	GGCTCACTTATGTGCCACTGTT	Exon 8		
FADS1_3851dn	GGACTCGTACTCTATACCATGCTTG	Exon 11	878	60°C
FADS1_4725up	TCCAGATTGAGCACCAGTGA	Exon 10/Intron 10		
FADS1_4726dn	TCCAAGGCTCTTCTTCCAGA	Exon 12	670	57°C (1x Q solution)
FADS1_4727up	GTTTTGAGGCTCCACTCTGC	Intron 11		
FADS1_4728dn	TGCTCACAGACCAGACCATC	3' UTR	388	60°C
FADS1_4729up	AGAGGGAGTCAGGAGGGTGT	3' flanking		
FADS1_4730dn	AGGACTCTGCTGGCTGTTGT	3' flanking	569	65°C (5% DMSO)
FADS2				
FADS2_4801up	GCACATCAGAGGTTCTGCAA	5' flanking		
FADS2_4802dn	GCAGGTTACGTGTCCGAACT	5' flanking	468	60°C
FADS2_5487up	CTGGAGGAAAGGCAAGGATA	Promoter		
FADS2_5488dn	GGGAAGAGCACAGGAGACAC	Intron 1	499	60°C
FADS2_4574up	TGGTGGAGACTGGCGGAGTT	Intron 1		
FADS2_4575dn	GTGGTCAGGGAGATGAGAGCA	Intron 2	519	60°C
FADS2_4803up	CTGAACTGGCTGTGGACAAA	Intron 2		
FADS2_4804dn	GTGCCCCACTGCTAACTGAT	Intron 4	643	60°C (1x Q solution)
FADS2_4805up	CAAGCTCAGGCATTCAGTCA	Intron 4		
FADS2_4806dn	GGAGGCTGCTAACACGCTAA	Intron 5	480	60°C
FADS2_4576up	TGACTCTGCTTTCTTGCTGTCC	Intron 6		
FADS2_4577dn	GATCTTGAGGGTTGGGTGCTA	Intron 7	273	60°C
FADS2_4807up	ACCTGCCTTACAACCACCAG	Exon 6		
FADS2_4808dn	CTGGACTCACCACCCAGTCT	Exon 7	491	60°C
FADS2_4860up	CTAGGGTTTCTTGGCGGAAG	Intron 7		
FADS2_4861dn	AGGAACCTGGAAGGGAGTGT	Intron 8/ Exon 9	426	55°C
FADS2_4578up	AAGGTTCAGGGCTCTCTTGC	Intron 8		
FADS2_4579dn	ACTGGTCCCTCCAAAAGGTC	Intron 9	440	60°C
FADS2_4580up	ACCCAAGGTCCATGTCTTCA	Intron 9		
FADS2_4581dn	TTGGTTAGTGCCCATCTTCC	Intron 10	458	60°C
FADS2_4582up	TCCCTTTATTGCCAGCGAAC	Intron 10		
FADS2_4583dn	GTCTCCTTTCAGCTCTCAACCAG	Intron 11	468	60°C
FADS2_4862up	CCTGGTTGAGAGCTGAAAGG	Intron 11		
FADS2_4863dn	GACCTCAAGAGAGGGTGGTG	3' UTR	510	63°C
FADS3				
FADS3_5327up	CTGTCAGCGCGGTTATAAGG	5' flanking		
FADS3_5328dn	AGGTCAAAGGTCAGCGAGAG	Intron 1	534	60°C
FADS3_5838up	GCAGCAGAAATTGACACAACA	Intron 1		
FADS3_5839dn	CAGGAAGAGACCGCAAACC	Intron 2	493	60°C
FADS3_5331up	TTGCTTCTGGGGATAACTGG	Intron 2		
FADS3_5332dn	TTAGGTGGCTGCAAGAAGTG	Intron 3	293	60°C
FADS3_5333up	GCCAAGCTGCATAAAAGAGG	Intron 3		
FADS3_5334dn	GAGGTGCTGGTGGTTGTAGG	Exon 6	770	60°C
FADS3_5840up	ATCTTCCACAAGGACCCAGA	Exon 5		

FADS3_5841dn	CCAGGAGAACAAGCAGACAC	Intron 6	406	60°C
FADS3_5335up	CTGTTCCTGAGCCCCTTCAC	Intron 6		
FADS3_5336dn	CTGCTACTCTCCCCTGCAAC	Intron 7	282	60°C
FADS3_5842up	AACCCAAGCCCAGAAGAGAC	Intron 7		
FADS3_5843dn	CCCATTCTACCGATGAGGAC	Intron 8	291	60°C
FADS3_5339up	CCCATCATACAGACGAGGAAG	Intron 8		
FADS3_5340dn	GGAAAGGCTGCTGAGAAAGG	Intron 11	764	60°C
FADS3_5341up	GAGAGAGCTGGGTGAAGGAG	Intron 11		
FADS3_5342dn	GGCTGGTTTAGCACAGGAAG	3' flanking	725	60°C
MLYCD				
MLYCD_4914up	TTAGATGGCGGGACAAAGAG	5' flanking		
MLYCD_4915dn	GGGGAGAAGCTGTCCTATCC	5' flanking	288	60°C
MLYCD_4960up	CGCTCATCATGAACTTGGAA	5' flanking		
MLYCD_4961dn	CTTGGCCATAGGAAGTCACC	5' flanking	666	60°C
MLYCD_5232up	TTCATTAGGCAGGGGTCAAG	Promoter		
MLYCD_5233dn	GCGACAGCTGCTAGGGATAC	Promoter	459	60°C
MLYCD_4916up	GCCCTAGAGGAGAGGGAATG	5' flanking		
MLYCD_4917dn	TGCTGATGTGATGGAAGAGG	Exon 1	732	60°C (1x Q solution)
MLYCD_4513up	CGCGGACTTTATGAGCTTCT	Exon 1		
MLYCD_4918dn	GCATGGGAAGATGGAGTGTT	Intron 1	448	60°C
MLYCD_4809up	AGCAAGTTCGTTGGTCAGGT	Intron 1		
MLYCD_4810dn	CAGAAAAGGGACACGTCACA	Intron 2	396	60°C
MLYCD_4967up	GCTCTTTGCAGACGCTCTCT	Intron 1		
MLYCD_4968dn	AGGGCTGGTTTTCATCTGTG	Intron 2	661	57°C
MLYCD_4811up	GCACGGTTTGTAACTCAGGA	Intron 2		
MLYCD_4812dn	GTCGTGCAGGTTTGGAGACT	Intron 3	376	60°C
MLYCD_4919up	GTGTGGCATCACCAGGACA	Intron 3		
MLYCD_4920dn	GGCGACTAGGCTGGAAGTC	Intron 4	664	60°C (1x Q solution)
MLYCD_4969up	GAGGGTGAAGGAGCACAGAG	Intron 3		
MLYCD_4970dn	ACTGGACAGTGGGAGCAAAG	Intron 4	654	62°C
MLYCD_4872up	GCTGAGGGAGGAGGAGTTTC	Intron 4		
MLYCD_4873dn	GGCGTGTTCTCCAGGAAGTA	Exon 5	526	57°C
MLYCD_4874up	GATCAACTGGATGGGTGACG	Exon 5		
MLYCD_4875dn	CTAAGCCAGGCAGGAAGATG	3' UTR	590	60°C
MLYCD_4958up	ATCCACGCACTGTCTCAGG	3' UTR		
MLYCD_4959dn	GCCTTCACCTCTGTCTCAGG	3' UTR	469	60°C
SCD1				
SCD1_3952up	AACTTCCCTAGTGCCCATCCT	5' flanking		
SCD1_3953dn	GGCTCAACTCTCTTCTACACCGA	Intron 1	877	60°C
SCD1_3985up	TGCGAGTGTTTTACCCCTCTAT	Intron 1		
SCD1_3986dn	GCGTTTCTACTCTGTGTAAGTTGC	Intron 2	623	60°C
SCD1_3954up	CCTGAAGGACACCTAGACGCT	Intron 2		
SCD1_3955dn	ATGGACCCAAGGACTGAACC	Intron 3	355	60°C
SCD1_3956up	AGTCCTGAGATTTGAAGGTGCT	Intron 3		
SCD1_3957dn	TGTCAGTTTCCCTGCTTATGTG	Intron 4	521	57°C
SCD1_3988up	TGTTGGGGATGGGAGCACTA	Intron 4		
SCD1_3989dn	CAGGCTACTGACATTCCGAGG	Intron 4	613	60°C
SCD1_3958up	TGCTCACAGCGTAGAGGGTAG	Intron 4		
SCD1_3959dn	AGAGAGGGGTCATAAAGCAGGT	Intron 5	433	60°C

SCD1_3960up	CCCTAACACAGGCTCACTCATA	Intron 5		
SCD1_3961dn	CGAAAGAATACTGTACTGGAACG	Exon 6	670	57°C
SCD1_3846up	AGCGTACTACCCTGACTATGGAT	3' UTR		
SCD1_3987dn	GGAATGCTGGTTAGTTTGCTG	3' UTR	699	60°C
SCD1_3990up	AGTGCTCACATTTGACGGAAG	3' UTR		
SCD1_3991dn	GCCTCAGAGAGAACCATAAAGATT	3' UTR	522	60°C
SCD5				
SCD5_4552up	TTCAGCCCTGGCAGTGGAAT	5' flanking		
SCD5_4553dn	CACCCAAGCCGATTGTGAAG	Intron 1	710	61°C (HotStart Taq + 1x Q solution)
SCD5_4554up	ACTGGCATCTGCTGACCTTC	Intron 1		
SCD5_4555dn	CACTCCCGAACATTTCACAG	Intron 2	347	60°C
SCD5_4556up	CCTGGATCGCTCTATTCCTCAG	Intron 2		
SCD5_4557up	ACAAGAAGACCCGACCGTCA	Intron 3	631	60°C
SCD5_5828up	ACACGACAGGGCACAAGAA	Intron 3		
SCD5_5829dn	CGAGCAGCCAAGAAGATGTG	Intron 4	380	60°C
SCD5_5830up	TCACACCTTTCCCTTCGACT	Exon 5		
SCD5_5831dn	TCTACCCTGCCTCTCTGCTC	Exon 5	558	60°C
SCD5_5832up	GATAGCCCGTTCCCTTTTTC	Exon 5		
SCD5_5833dn	TTTGATGTCTGGCACCTCTG	Exon 5	415	60°C
SCD5_5834up	CAGAGGTGCCAGACATCAAA	Exon 5		
SCD5_5835dn	GGCGTCATCTCGTTCTCAA	Exon 5	465	60°C

Genotyping

Genotypes of nine sequence variants (Table 3.9) in the M × P family and the breed panel were determined by PCR, PCR-RFLP and TaqMan genotyping assays (Table 3.4). Furthermore, the known C1843T mutation in porcine *RYR1* (Fujii *et al.* 1991) was genotyped in the M × P family using PCR-RFLP (Table 3.4). The haplotypes were determined using PHASE version 2.1 (Stephens *et al.* 2001; Stephens & Donnelly 2003).

Table 3.4 PCR, PCR-RFLP and TaqMan assays used to genotype the pig populations.

Genotyping method	Gene symbol	Primer pairs[1]	Polymorphisms	Location	Restriction enzyme	Unit per reaction	Reaction temperature
PCR	*ACACA*[2]	4962 4963	g.227756_ 227757ins280	Intron 44			
PCR-RFLP	*ACACB*	5041 5042	g.25835A>G (p. Ser86Gly)	Exon 2	*Cfr10*I	0.5	37°C
	ACACB	5041 5042	g.25926G>A (p. Ser116Asn)	Exon 2	*Tai*I	0.5	65°C
	ACACB	5041 5042	g.26053G>A	Exon 2	*Pvu*II	0.5	37°C
	FADS2	4805 4806	g.28505G>A	Intron 5	*Hha*I	0.5	37°C
	MLYCD	4872 4873	g.15996G>A (p. Gly388Asp)	Eoxn 5	*Aas*I	0.5	37°C
	SCD1	3846 3987	g.16663T>C	3' UTR	*Msp*I	0.5	37°C
	RYR1	104 105	c.1843C>T (p.Arg615Cys)		*HhaI*	2.0	37°C

Genotyping in ABI 7500 Fast Real-time PCR System (Applied Biosystems)

	Gene symbol	SNPs	Location	TaqMan assay primers and MGB probe sets
TaqMan assay genotyping	*ACACB*	g.20124A>G	Promoter II	ACACB-1570F: GCTGGTCCAGGCTCCTC ACACB-1570R: CACAGGGTGACTAAGGGAAGTG ACACB-1570A: ATGGTGCATC**A**TACCAG (VIC-labelled) ACACB-1570G: TGGTGCATC**G**TACCAG (FAM-labelled)
	CPT2	g.41033G>A (p. Val397Ile)	Exon 4	CPT2-1549F: CGTGCTCAGGTTCTTAAATGAAGTG CPT2-1579R: CGCGGGACGAGTCAGT CPT2-1549A: CCCTGCC**A**TCACTC (VIC-labelled) CPT2-1549G: CCTGCC**G**TCACTC (FAM-labelled)

[1]Primer sequences are shown in Table 3.3.

Primer_104 forward: 5'-GTTCCCTGTGTGTGTGTGTGTGCAAT-3'

Primer_105 reverse: 5'-CTGGTGACATAGTTGATGAGGTTTG-3'.

[2]Genotypes can be distinguished using PCR analysis due to the fragment difference resulting from the deletion and insertion of 280 bp.

Statistical analysis

Statistical analysis was performed using R language and environment extended by R packages (http://www.r-project.org/). Distribution of the lipid-related phenotypes in the M × P F_2 population (Table 3.1) was examined and an outlier test was performed using Grubbs test in the package 'outliers' (version 0.13, Komsta 2006). BFT at mid-back (BFTM) and IMF were Box-Cox and natural logarithm transformed, and n-3PUFA and n-6PUFA square root transformed because of their skewed distribution. Outliers were removed ($P < 0.01$) if still detected after the data transformation.

A linear model was used to estimate the relationship between candidate gene genotype and the lipid-related traits. The model accounted for the fixed effects: genotype, gender, father and mother. Live weight at slaughtering was used as a covariate for BFT traits, BFTM for IMF and IMF for fatty acid composition. Additionally, the effect of the *RYR1* C1843T genotypes was taken into account when significant. The nominal *P*-values for candidate gene genotypes were obtained by ANOVA analysis in the package 'car' (version 1.1-1, Fox 2006). Least squares means (± SE) for candidate gene genotypes were estimated with the untransformed data based on the respective model using the package 'effects' (version 1.0-8, Fox 2005).

Results

Genomic structure

Genomic structures for the candidate genes except for *SCD1* have not been previously described in pigs. Therefore, twelve BACs were identified for ten uncharacterized genes using the *in silico* screening approach combined with colony PCR tests (Table 3.5). The BACs were not completely sequenced, but to an extent that assembled contigs containing the target genes could be identified based on BLAST analysis using human mRNA sequences (Table 3.5). The average coverage of consensus sequences obtained from each BAC ranged from 3.99 to 14.87-fold. The identified genomic contigs have been deposited in GenBank (Table 3.5). They were subject to cDNA/EST-based gene prediction using the *GenomeThreader* software. Genomic structures were elucidated based on the predictions and manual editing using the Apollo tool (Fig. 3.1). The ten genes considerably differ not only in genomic size ranging from ~7.8 kb (*CPT1B*) to > 292 kb (*ACACA*), but also in the number of exons from five (*MLYCD*) to more than 50 (*ACACA*). Most predicted

intron/exon boundaries follow the GT-AG rule. Putative non-canonical splice sites GC-AG were found in intron 43 of *ACACA* and intron 11, 12, 23 and 37 of *ACACB*, and GG-AG in intron 38 of *ACACB*. Each protein sequence derived from porcine gene annotation was compared with its counterpart in humans, cattle and rodents respectively. There was a high degree of sequence identity (81.2 % - 98.9 %) between pigs and other mammals (Table 3.5).

Figure 3.1 Genomic structures of eleven porcine genes involved in lipid metabolism (see next page). Coding and untranslated regions are represented by ■ and □ respectively. Sizes for exons and introns (in bp) are shown. The numbers in parentheses on the left side are the predicted gene sizes, and the numbers under the gene structures are the sizes of coding regions in exons. The question marks indicate unknown gaps between the contigs. The alternative splicing of human *ACACA* (only three main splicing patterns listed here, for all see Mao *et al*. 2003) and *ACACB* (Barber *et al*. 2005; Oh *et al*. 2005a) in 5' end using different promoters is indicated with dotted lines. The presence of porcine-specific exon 3 (▨) is unknown in *ACACA*.

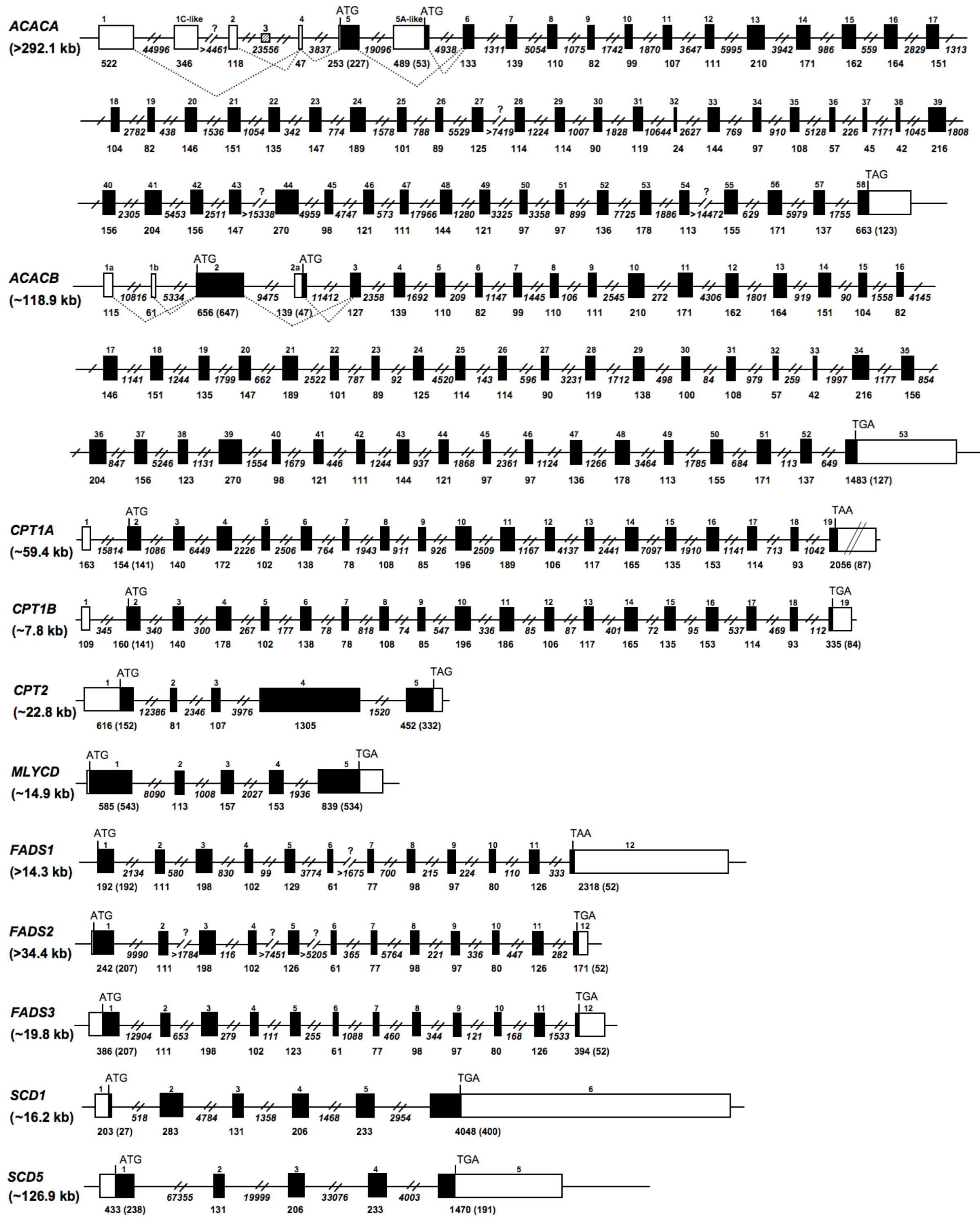
ACACA
(>292.1 kb)
ACACB
(~118.9 kb)
CPT1A
(~59.4 kb)
CPT1B
(~7.8 kb)
CPT2
(~22.8 kb)
MLYCD
(~14.9 kb)
FADS1
(>14.3 kb)
FADS2
(>34.4 kb)
FADS3
(~19.8 kb)
SCD1
(~16.2 kb)
SCD5
(~126.9 kb)

Table 3.5 Porcine and human gene information, and protein sequence identity between pigs and other mammals.

Function category	Gene symbol	Porcine gene information				Human mRNA acc. no.	Protein sequence identity (%)[d]			
		BACs	SSC	Genomic sequence acc. no.	Protein (resid-ues)[c]		Pig-Human %, acc. no.	Pig-Cattle[e] %, acc. no.	Pig-Rat %, acc. no.	Pig-Mouse %, acc. no.
Fatty acid synthesis	*ACACA*	CH242-27L18 RP44-262I23 PigE-185P4	12[a]	FJ263680	2346	NM_198839	97.9% NP_942136	98.9% NP_776649	98.1% NP_071529	98.5% NP_579938
Fatty acid oxidation	*ACACB*	RP44-265J11	14[b]	EU853705	2454	NM_001093	88.7% NP_001084	90.1% XP_873014	82.4% NP_446374	85.7% NP_598665
	CPT1A	PigE-148I15	2[b]	FJ263681	772	NM_001876	89.4% NP_001867	-	86.0% NP_113747	85.9% NP_038523
	CPT1B	CH242-132N13	?	FJ263682	772	NM_004377	89.4% NP_004368	91.3% NP_001029521	85.7% NP_037332	86.9% NP_034078
	CPT2	CH242-219C5	6[b]	FJ263683	658	NM_000098	84.0% NP_000089	84.5% NP_001039354	80.4% NP_037062	82.5% NP_034079
	MLYCD	CH242-437N5	6[b]	FJ263687	499	NM_012213	84.0% NP_036345	87.6% NP_001092416	81.2% NP_445929	82.2% NP_064350
Fatty acid desaturation	*FADS1*	CH242-222K18	2[b]	FJ263684	440	NM_013402	88.4% NP_037534	89.0%	87.9% NP_445897	87.1% NP_666206
	FADS2	CH242-222K18	2[b]	FJ263685	444	NM_004265	86.9% NP_004256	90.8% NP_001076913	87.4% NP_112634	86.7% NP_062673
	FADS3	PigE-219E3	2[b]	FJ263686	443	NM_021727	90.3%, 445 NP_068373	93.7%, 443 NP_001077160	90.0%, 449 NP_775160	90.6%, 449 NP_068690
	SCD1[f]	-	14[a]	AY487830	359	NM_005063	86.9%, 359 NP_005054	88.3%, 359 NP_776384	85.8%, 358 NP_631931	84.4%, 355 NP_033153
	SCD5	PigI-285B6 CH242-305N1	8[b]	FJ263688	332	NM_001037582	89.5 %, 330 NP_001032671	91.6 %, 335 NP_001070413	-	-

[a]*ACACA* was mapped by Calvo *et al.* (2000) and *SCD1* by Ren *et al.* (2003).

[b]The location of BACs identified for specific genes on the Porcine Genome Physical BAC map (http://www.sanger.ac.uk/Projects/S_scrofa/mapping.shtml).

[c]Proteins for *ACACA* and *ACACB* were predicted using the start codon in exon 5 and exon 2, respectively and for *MLYCD*, using the first in-frame start codon.

[d]Percent identities were calculated by the ExPASy SIM Alignment Tool for protein sequences using the BLOSUM62 comparison matrix with a Gap Open penalty of 12 and a Gap Extension penalty of 4.

[e]Bovine FADS2 protein sequence was obtained based on the annotation of bovine *FADS2* using the sequence from Btau_4.0, but bovine CPT1A protein sequence could not be predicted due to incomplete genomic sequence.

[f]Porcine *SCD1* genomic sequence (AY487830) is publicly available. No BAC was identified for the gene.

?: The localization of *CPT1B* could not be predicted due to the absence of CH242-132N13 on the Porcine Genome Physical BAC map.

ACACA - Porcine *ACACA* comprises 54 coding exons (exon 5 - 58, Fig. 3.1) as in human *ACACA* (Mao *et al*. 2003). The predicted organization of the coding exons was further validated by the recently released porcine mRNA sequence (NM_001114269). Because few 5' porcine ESTs were available and no analysis of 5’ end has been performed so far, putative extents of exons in 5’ end of porcine *ACACA* were mainly defined by homology with exons in human *ACACA*, in which the organization of 5’ region has been extensively investigated (Mao *et al*. 2003). There are three leader exons (exon 1, exon 2 and exon 5A) used by promoter PI, PII and PIII, respectively and seven additional exons (exon 1A, 1B, 1C, 3, 4, 5A' and 5B) used for alternative splicing in the 5’ end of human *ACACA*. In contrast to PI and PII transcripts with the start codon in exon 5, PIII transcripts contain the start codon in exon 5A, generating an isoform, in which a N-terminus of 75 amino acids encoded by exon 5 is replaced by 17 residues encoded by exon 5A (Barber *et al*. 2005).

Among ten exons found in 5’ end of human *ACACA*, exon 1, 1C, 2, 4 and 5A showed homology with the porcine *ACACA* genomic sequence (FJ263680). The presence of porcine exon 1 was also supported by 5' RACE (data not shown), exon 2 by an EST (AJ684616) and exon 4 by the mRNA sequence (NM_001114269). Although hits of human exon 1C and 5A on the porcine sequence (designated as exon 1C-like and 5A-like in Fig. 3.1) were found, no porcine ESTs containing them have been identified to date. Further analysis is required to address whether pigs express transcripts containing exon 1C-like or 5A-like. No homology between human exon 3 and the porcine sequence seems expected because there is no homology among human, rat (Luo *et al*. 1989) and bovine (Mao *et al*. 2001) exon 3, indicating that it might be unique to each species. Thus, it remains to be elucidated whether a pig-specific exon 3 exists. No porcine genomic region showed homology with human exon 1A, 1B, 5A’ or 5B. Exon 1A and 1B lie between exon 1 and 1C in human *ACACA*, and exon 5A’ and 5B between exon 5 and 6. Transcripts containing 1A, 1B, 5A’ or 5B were not identified in cattle and rats (Mao *et al*. 2003). In conclusion, porcine *ACACA* contains at least 57 exons (exon 1, 2, 4 and 5-58), and has a genomic size (at least 292.1 kb) comparable to human *ACACA* (~330 kb), which has 64 exons.

ACACB - The genomic structure of porcine *ACACB* is similar to that of human *ACACB* (Barber *et al.* 2005). Both *ACACB* comprise 52 coding exons (exon 2 - 53, Fig. 3.1). Human *ACACB* contains at least two promoters (PI and PII), generating transcripts distinct in 5' UTRs (designated as exon 1a and 1b, respectively) but sharing the remaining exons after splicing (Oh *et al.* 2005a). The two transcript variants encode the same protein because both use the same start codon in exon 2 for translation initiation. Although the computer-based prediction captured neither exon 1a nor 1b in the porcine genomic sequence (EU853705), their presence was validated by RT-PCR (data not shown). The existence of a third *ACACB* promoter was proposed on the basis of a cDNA sequence (CA392208) in humans (Barber *et al.* 2005). PIII was suggested to initiate a transcript variant containing a unique exon, therefore generating a 15 amino acid variant N-terminus. A homologous region to this exon was found in the porcine sequence (annotated as exon 2a in Fig. 3.1). In conclusion, porcine *ACACB* consists of 55 putative exons and spans approximately 118.9 kb.

CPT1A - Porcine *CPT1A* spans ~59.4 kb and a 2319-bp putative coding region comprises 18 exons (exon 2 - 19, Fig. 3.1). Sizes of coding region in these exons are highly conserved between humans and pigs except for exon 9, which has a size of 85 bp in porcine *CPT1A*, but 88 bp in human *CPT1A* (Gobin *et al.* 2002). Manual annotation of 5' end in porcine *CPT1A* revealed potential alternative splicing. Two distinct groups of porcine ESTs that indicated two possibilities of alternative splicing have been identified so far. The first group (BW983716 and BW973449) supports the existence of a non-coding exon (exon 1) followed by exon 2 containing the start codon (shown in Fig. 3.1). The second group (DY410467 and DY411500) supports the existence of exon 2 with a larger size because DY410467 with longer 5’ end contains a 47-bp untranslated sequence that is different from exon 1 but has perfect match with the 47-bp genomic sequence directly upstream of exon 2. In human *CPT1A*, two non-coding exons (exon 1a and 1b) were found, which could be alternatively spliced to exon 2 using two different promoters (Gobin *et al.* 2002). Comparison analysis demonstrated that porcine exon 1 shows homology to human exon 1b. Likewise, possible alternative splicing was found in 3' end of porcine *CPT1A*. Porcine ESTs (DB790591 and DB782191) and a cDNA sequence (AF288789) provide evidence for the existence of an additional non-coding exon downstream of porcine exon 19 containing the stop codon. However, it was impossible to define the exact border of intron between them because no consensus splice sites could be identified in the predicted intron-exon

boundaries. The other EST group (DY408766, CO994928 and BF708902) supports the presence of a long and uninterrupted untranslated region after the stop codon (Fig. 3.1). In human *CPT1A*, no evidence was obtained for the presence of non-coding exon in the 3' end (Gobin *et al*. 2002).

CPT1B - Porcine *CPT1B* is composed of 19 exons distributed over an approximately 7.8-kb genomic region (Fig. 3.1). Similar to human *CPT1B* (Yamazaki *et al*. 1997), porcine *CPT1B* contains 18 coding exons (exon 2 - 19). The derived coding region shows 99% sequence identity with that in the porcine *CPT1B* mRNA sequence (NM_001007191). Human *CPT1B* contains two alternatively transcribed first non-coding exons (exon 1A/U and exon 1B/M) resulting from the use of two promoters (van der Leij *et al*. 2002). Sequence comparison revealed that porcine exon 1, whose existence is evidenced by the EST (AW430779), contains the equivalent of human exon 1B (M) and part of the genomic sequence separating exon 1A and exon 1B. An additional non-coding exon 20 after exon 19 was reported in human *CPT1B* (van der Leij *et al*. 2002). However, ten currently available porcine 3' ESTs do not support the existence of such a non-coding exon in pigs.

CPT2 – Similar to human *CPT2* (Verderio *et al*. 1995), porcine *CPT2* contains five exons, which greatly vary in size ranging from 81 bp (exon 2) to 1305 bp (exon 4). The gene spans approximately 22.8 kb (Fig. 3.1).

MLYCD - Porcine *MLYCD* consists of five exons and four introns, and spans approximately 15 kb (Fig. 3.1). The exon/intron organization of porcine *MLYCD* resembles that of human *MLYCD* (Wightman *et al*. 2003). Two functional in-frame ATG sequences suggested in human exon 1 are conserved in porcine exon 1. The first ATG in porcine *MLYCD* (Fig. 3.1) is located 129 bp upstream of the second one (not shown). Therefore, the resultant protein sequence (499 residues) using the first ATG is 43 amino acids longer than that using the downstream ATG.

FADS1*, *FADS2* and *FADS3 – Three porcine *FADS* genes share similar gene structure, which all contain 12 exons and 11 introns (Fig. 3.1). The coding region of each gene comprises all 12 exons. Furthermore, they have an identical length in exon 2 - 4, exon 6 - 11 and the coding region of the last exon. There is a minor difference in size of exon 5 and the coding part of first exon among them. However, they greatly differ in intron size, reflecting differences in genomic size: *FADS1* (>14.3 kb), *FADS2* (>34.4 kb) and *FADS3* (~19.8 kb). The remarkable similarity of the exon/intron organization among the three *FADS* genes in pigs is consistent with the findings in humans (Marquardt *et al*. 2000).

SCD1* and *SCD5 - Like human and bovine *SCD5* (Wang *et al*. 2005; Lengi & Corl, 2007), porcine *SCD5* consists of five exons and four introns (Fig. 3.1). Porcine *SCD5* spans about 126.9 kb. The putative coding sequence of porcine *SCD5* (999 bp) shows 100% identity with that derived from the porcine mRNA sequence (NM_001114278). In contrast to porcine *SCD5*, porcine *SCD1*, whose genomic structure was described by Ren *et al.* (2004b), is composed of six exons and five introns and has a much smaller gene size of approximately 16.2 kb (Fig. 3.1).

Amino acid sequence features

ACC proteins - The putative protein encoded by porcine *ACACA* with ATG in exon 5, which is composed of 2346 residues, exhibits a high degree of sequence identity (97.9% - 98.9%) to that of cattle, human, rat and mouse (Table 3.5). The high conservation of ACACA proteins in mammals underscores the importance of ACACA biological functions. The deduced ACACB protein of 2454 residue shows 82% - 90% sequence identity with human, mouse, rat and bovine counterparts (Table 3.5). The conserved domain prediction showed that porcine ACACA and ACACB belong to complex multifunctional enzyme systems and that both comprise three functional domains (Fig. 3.2). Two functional domains: biotin carboxylase (BC) and biotin carboxyl carrier protein (BCCP), reside in the N-terminal of one-third of the proteins. The third domain: carboxyltransferase (CT) domain is located in the C-terminal of one-third of the protein (Fig. 3.2). The highly conserved ACC central region, whose function is still unknown, separates BC-BCCP domains and CT domain. Both ACCs are biotin-containing proteins and the site of biotin attachment (Lys786 in porcine ACACA and Lys925 in porcine ACACB, Fig. 3.2) is absolutely conserved in mammals (Barber *et al*. 2005).

Porcine ACACA and ACACB proteins resemble each other closely in the structure organization, but they greatly differ in the N-terminal region. The N-terminus of porcine ACACB protein is 108 amino acids (aa) longer than that of porcine ACACA (Fig. 3.2). Similar to human ACACB protein, the first N-terminal 20 aa of porcine ACACB are characteristic of high hydrophobicity based on the hydrophobicity analysis at website: http://www.vivo.colostate.edu/molkit/hydropathy/. The first N-terminal 20 aa in humans was suggested to target the ACACB protein to mitochondria where regulation of fatty acid oxidation takes place through the inhibition of carnitine palmitoyltransferase 1 by ACACB's product: malonyl-CoA (Abu-Elheiga *et al*. 2000). The lack of such hydrophobic segment in ACACA agrees with the fact that ACACA is a cytosolic protein. Four conserved phosphorylation sites (corresponding to rat Ser29, Ser79, Ser1200 and Ser1215, Barber *et al*. 2005) in porcine ACACA and one (corresponding to rat Ser218, Barber *et al*. 2005) in porcine ACACB are shown in Fig. 3.2.

CPT proteins - The putative porcine CPT1A and CPT1B proteins show 85.9% - 91.3 % sequence identity with their respective counterparts in humans, rats, mice and cattle (Table 3.5). In addition, porcine CPT1A protein is 62.3% homologous to porcine CPT1B. Both CPT1 isoforms are integral proteins of the outer mitochondrial membrane (OMM). The first 150 N-terminal residues of the rat liver CPT1A were found to specify both mitochondrial targeting and anchorage at the OMM (Cohen *et al*.1998; Cohen *et al*. 2001). They contain two transmembrane (TM1 and TM2) segments corresponding to rat CPT1A residues 48-75 and residues 103-122 respectively and an internal mitochondrial import signal (residues 123-147) located immediately downstream of TM2 (Fig. 3.2). The mitochondrial import signal specifies mitochondrial targeting, whereas the hydrophobic TM segment(s) acts as a stop-transfer sequence that stops and anchors the translocating CPT1 into the OMM (Cohen *et al*.1998; Cohen *et al*. 2001). The catalytic His473 residue in the C-terminal domain is absolutely conserved in CPT1s in the mammals (Fig. 3.2).

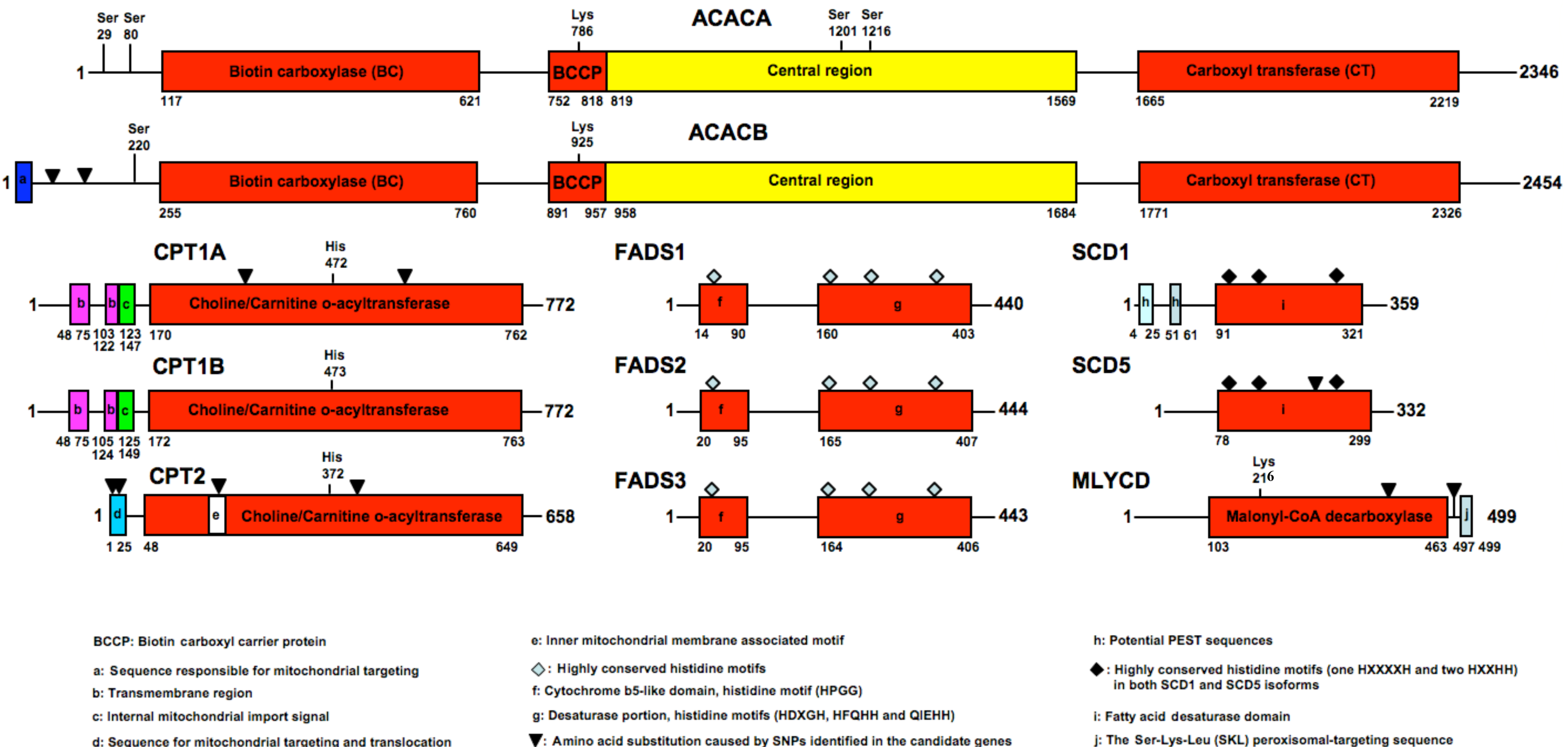

Figure 3.2 Putative functional regions of the proteins encoded by eleven porcine genes involved in lipid metabolism. The functional domains in the porcine putative proteins were predicted using the Conserved Domain Architecture Retrieval Tool (CDART, http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Additional protein features suggested in other mammals (see the text in the part ‘amino acid sequence feature’) are also shown.

Unlike CPT1 proteins, CPT2 is loosely associated with the surface of the inner mitochondrial membrane (IMM). Consistent with the subcellular localization, rat CPT2 contains a 25-residue region in the N-terminus (residues 1-25), which is crucial for its mitochondrial targeting and translocation across both mitochondrial membranes (Brown *et al*. 1991), and an IMM associated motif (residues 179-208, Hsiao *et al*. 2006). Upon mitochondrial import, rat precursor targeting peptide of 658 residues is cleaved between leucine 25 and serine 26, and therefore is changed into a mature protein of 633 residues without the 25-residue N-terminal mitochondrial targeting signal (Brown *et al*. 1991). The catalytic residue His372 suggested in Hsiao *et al*. (2006) is highly conserved in all CPT2 members (Fig. 3.2).

FADS proteins - The fatty acid desaturase genes (*FADS*1-3) are thought to have arisen evolutionarily from gene duplication based on their similar exon/intron organization. Therefore, it is anticipated that high homology exists between porcine FADS1 protein and FADS2 (63.6%), FADS1 and FADS3 (54.9%), and FADS2 and FADS3 (61.5%). In addition, there is a high degree of sequence identity between porcine FADS proteins (FADS1-3) and their respective orthologs in other mammals (Table 3.5). FADS family members are considered fusion products composed of an N-terminal cytochrome b5-like domain and a C-terminal multiple membrane-spanning desaturase portion, both of which are characterized by conserved histidine motifs (Fig.3.2).

Three highly conserved histidine-rich motifs (HDXGH, HFQHH and QIEHH) exist in the desaturase domain, which were found to be essential to the catalytic center of desaturases (Nakamura & Nara, 2004). The highly conserved histidine rich motif (HPGG), characteristic of the cytochrome b5-like domain, was found to be required for rat FADS2 enzyme activity (Guillou *et al*. 2004). In addition, rat FADS2 was shown to localize in the endoplasmic reticulum. Analysis of transmembrane topology of porcine FADSs (http://www.vivo.colostate.edu/molkit/hydropathy/) identified four putative transmembrane domains in each FADS member, which indicated that they are potential membrane-associated proteins.

MLYCD protein - The MLYCD proteins in mammals are highly conserved (Table 3.5). The lysine residue at position 210 (K210) in rat MLYCD was shown to be essential for its catalysis (Nam *et al*. 2006). This highly conserved lysine residue is located in the decarboxylation domain in porcine (residue 216, Fig. 3.2), human (residue 210) and mouse (residue 210) MLYCD. However, a chemically similar arginine residue (R216) appears in the corresponding site in bovine MLYCD. Subcellular localization analysis revealed MLYCD resides in cytoplasm and peroxisomes (Sacksteder *et al*. 1999). In agreement with the subcellular distribution in peroxisomes, a highly conserved peroxisome targeting motif (Ser-Lys-Leu_{COOH}) in the very C-terminal part was found in porcine MLYCD (Fig. 3.2).

SCD proteins - The predicted amino acid sequence for porcine SCD5 contains 332 residues. Four SCD isoforms (SCD1, 2, 3 and 4) have been identified in mice, but two in rats (SCD1 and SCD2), and two in humans and cattle (SCD1 and SCD5). Porcine SCD5 shows 91.6 % and 89.5 % sequence identity with bovine and human SCD5 respectively, whereas it demonstrates about 60.9-65.1% with SCD1, SCD2, SCD3 and SCD4 in different mammals. So far, only one SCD gene (*SCD1*) in pigs has been characterized. Porcine SCD1 shares only 65.9% sequence identity with porcine SCD5. Taken together, these comparisons indicate that we have characterized the porcine ortholog of human and bovine *SCD5*. Hydrophobicity analysis of porcine SCD5 (http://www.vivo.colostate.edu/molkit/hydropathy/) revealed four putative transmembrane domains. The characteristic resembles that of other SCD isoforms *(*Man *et al.* 2006*)* as well as the FADS members (see above). In addition, three histidine motifs (one HXXXXH and two HXXHH), which are catalytically essential to acyl-CoA desaturases (Shanklin *et al.* 1994), are conserved in porcine SCD5 (Fig. 3.2). Compared to porcine SCD1, no N-terminal PEST sequences were found in porcine SCD5 (Fig. 3.2). PEST sequences are characterized by having high local concentrations of amino acids proline (P), glutamic acid (E), serine (S), threonine (T) and to a lesser extent aspartic acid (D). The PEST sequences were found to be responsible for rapid degradation of SCD1 (Mziaut *et al*. 2000).

Identification of sequence variants

All putative exons, small introns, intron-exon boundaries in *CPT1A*, *CPT1B*, *CPT2*, *MLYCD*, *FADS1*, *FADS2*, *FADS3*, *SCD1* and *SCD5* were screened with some exceptions: an attempt to re-sequence exon 1 and 2 in *CPT1A* failed due to the high GC content, and 3 ' UTR in *FADS1*, *SCD1* and *CPT1A* were only partly investigated. Furthermore, 2 kb upstream of the start codon in *MLYCD* and *CPT2* were analyzed. Both *ACACA* and *ACACB* contain more than 50 exons. Thus, priority of screening was given to exons encoding three functional domains: biotin carboxylase, biotin carboxyl carrier protein and carboxyltransferase (Barber *et al*. 2005). A total of 38 exons in *ACACA* and 48 exons in *ACACB* were selected for polymorphism detection (Table 3.6).

Table 3.6 Exons selected for sequence variant detection in porcine *ACACA* and *ACACB*.

Structure unit[1]	Gene symbol			
	ACACA		*ACACB*	
	Exons encoding the structure unit	Exons resequenced	Exons encoding the structure unit	Exons resequenced
BC	Exon 6 - 17	Exon 6 - 17	Exon 3 - 14	Exon 3 - 14
BCCP	Exon 21 - 22	Exon 21 - 22	Exon 18 - 19	Exon 18 - 19
Central region	Exon 22 - 42	Exon 23, 26, 27, 41	Exon 19 - 37	Exon 23, 24, 25, 26, 29, 30, 31, 34, 36, 37
CT	Exon 44 - 56	Exon 44 - 56	Exon 39 - 51	Exon 39 - 51
Others		Exon 1, 4, 18, 19, 20, 57, 58		Exon 1a, 1b, 2, 15,16,17, 52, 53

[1]BC: Biotin carboxylase, BCCP: Biotin carboxyl carrier protein, CT: Carboxyltransferase, the central region separates BC-BCCP and CT domains.

In total, 85.5 kb in the eleven genes were re-sequenced using twelve parental animals of the M × P cross and 367 sequence variants were identified including 359 SNPs, six insertion-deletion polymorphisms and two microsatellites (Table 3.7). The overall distribution of the sequence variants was one every 233 bp. Porcine *ACACA* was recently screened by cDNA re-sequencing (Munoz *et al*. 2007) and porcine *SCD1* by genomic re-sequencing (Ren *et al*. 2004b). Comparison of our data with the reported variants in *ACACA* (EF618729) and *SCD1* (AY487830) demonstrated that eight variants in *ACACA* and three in *SCD1* have been previously described. Therefore, our study reveals 356 novel sequence variants. The sub-regional distributions of all sequence variants are as follows: 37 in 5' end, 219 in introns, 83 in the coding elements and 28 in 3' end (Table 3.7). Of the 83 SNPs in the coding regions, eleven cause amino acid exchanges including two in *ACACB* (not located in

the aforementioned conserved domains), two in *CPT1A*, four in *CPT2*, two in *MLYCD* and one in *SCD5* (Table 3.8). Among them, three neutral to acidic (negatively charged) residue substitutions and one neutral to basic (positively) were found. Three of the eleven substitutions occurred at residues that are invariant across different species. However, prediction using the online tool PolyPhen (Ramensky *et al*. 2002) revealed that effects of all the eleven non-synonymous SNPs on protein structure and function are benign.

Table 3.7 Summary of sequence variants found in eleven genes involved in lipid metabolism.

Gene symbol	Base pair screened (kb)	Total number of variants	Number of variants by type			Number of variants in non-coding regions			Number of SNPs in coding regions		Frequency (bp/variant)
			SNP	INS/DEL	MS[1]	5' end[2]	Intron	3' end[2]	Synonymous	Non-synonymous	
ACACA	17.6	62	60	1	1	0	38	8	16	0	284
ACACB	18.9	104	104	0	0	7	69	3	23	2	182
CPT1A	6.8	38	37	1	0	0	32	0	4	2	179
CPT1B	7.3	4	4	0	0	1	2	0	1	0	1825
CPT2	4.8	25	25	0	0	10	5	0	6	4	192
MLYCD	5.1	68	65	2	1	13	33	7	13	2	75
FADS1	6.4	17	17	0	0	1	13	1	2	0	376
FADS2	5.7	21	21	0	0	4	16	0	1	0	271
FADS3	4.2	10	10	0	0	0	7	0	3	0	420
SCD1	5.3	4	4	0	0	0	2	2	0	0	1325
SCD5	3.4	14	12	2	0	1	2	7	3	1	243
Total	**85.5**	**367**	**359**	**6**	**2**	**37**	**219**	**28**	**72**	**11**	**233**

[1]Microsatellites.

[2]The 5’ end includes 5’ UTR and its upstream region, and the 3’ end contains 3’ UTR and its downstream sequence.

Table 3.8 Amino acid substitutions caused by eleven non-synonymous SNPs found in five genes.

Gene symbol	Non-synonymous SNPs	Location	Codon change	Protein residue	Amino acid property change	Residue conservation across 5 species[1]
ACACB	EU853705: g.25835A>G	Exon 2	AGC/GGC	Ser86Gly	Polar, uncharged / nonpolar, uncharged	Variable
	EU853705: g.25926G>A	Exon 2	AGC/AAC	Ser116Asn	No change	Invariant
CPT1A	FJ263681: g.95956A>G	Exon 10	CAG/CGG	Gln328Arg	Polar, uncharged / positively charged	Variable
	FJ263681: g.106837A>G	Exon 14	AAC/AGC	Asn537Ser	No change	Variable
CPT2	FJ263683: g.21149A>G	Exon 1	ATG/GTG	Met2Val	No change	Variable
	FJ263683: g.21183A>G	Exon 1	GAC/GGC	Asp13Gly	Negatively charged / nonpolar, uncharged	Variable
	FJ263683: g.40451G>A	Exon 4	GGC/AGC	Gly203Ser	Nonpolar, uncharged / polar, uncharged	Invariant
	FJ263683: g.41033G>A	Exon 4	GTC/ATC	Val397Ile	No change	Variable
MLYCD	FJ263687: g.15996G>A	Exon 5	GGC/GAC	Gly388Asp	Nonpolar, uncharged / negatively charged	Variable
	FJ263687: g.16238G>A	Exon 5	GCC/ACC	Ala469Thr	Nonpolar, uncharged / polar uncharged	Variable
SCD5	FJ263688: g.148175G>A	Exon 4	GAC/AAC	Asp253Asn	Negatively charged / polar, uncharged	Invariant

[1]Proteins from five species, namely pigs, humans, rats, mice and cattle were compared to examine the conservation of the residues where substitutions occurred. The protein sequences used were listed in Table 3.5. Because no SCD5 existed in rodents, only proteins from pigs, humans and cattle were aligned.

Association study

An association study was conducted between nine variants in six candidate genes (Table 3.9) and the lipid-related traits (fatness, IMF and fatty acid composition) in approximately 580 pigs of the M × P F_2 population (Table 3.1). Allele frequencies observed in the F_2 population and in the four pig breeds are shown in Table 3.9. The results indicate that most of the studied variants were segregating in the four commercial breeds (Table 3.9). In addition, five haplotypes were constructed from four genotyped SNPs in *ACACB* (Table 3.9). Four of them were observed in the F_2 population, namely [A; A; A; A], [A; A; G; G], [A; G; A; G] and [G; A; G; G] with a frequency of 40%, 5%, 11% and 44% respectively. At least three of the five haplotypes were present in each commercial breed. Moreover, the haplotype distribution and frequency greatly differed between these breeds (Table 3.9).

Five variants in *ACACA*, *ACACB*, *FADS2* and *SCD1* showed associations with several lipid-related traits at $P < 0.1$ in the F_2 population (Table 3.10). However, none of these associations remained significant after Bonferroni correction for 108 (12 traits × 9 variants, Table 3.1 and Table 3.9) tests at a threshold of 4.6×10^{-4} (0.05/108). The tested variants in *CPT2* and in *MLYCD* showed no association reaching the $P < 0.1$ threshold (data not shown). In this study, no association analysis was performed for five remaining genes (*CPT1A*, *CPT1B*, *FADS1*, *FADS3* and *SCD5*) for the following reasons. No obviously functional variants could be identified in *FADS1* and *FADS3*. Although some interesting variants (e.g. promoter SNPs or non-synonymous SNPs) were found in *CPT1A*, *CPT1B* and *SCD5*, they were not highly informative in the M × P family. Therefore, further investigation of these five genes in other populations is needed to clarify their effects on the lipid-related traits.

Table 3.9 Allele frequencies of nine variants in *ACACA*, *ACACB*, *CPT2*, *MLYCD*, *FADS2* and *SCD1*, and *ACACB* haplotype distribution.

Gene (allele)[1]	Sequence variants/Haplotypes	Location	DE	DL	DU	PI	M × P F_2 cross
ACACA (del)	FJ263680: g.227756_ 227757ins280	Intron 44	0.17	0.31	0.00	0.39	0.30
ACACB (A)	EU853705: g.20124A>G	Promoter II	1.00	0.98	1.00	0.91	0.55
ACACB (A)	EU853705: g.25835A>G (p. Ser86Gly)	Exon 2	0.47	0.58	0.30	0.62	0.89
ACACB (A)	EU853705: g.25926G>A (p. Ser116Asn)	Exon 2	0.70	0.91	0.93	0.90	0.51
ACACB (A)	EU853705: g.26053G>A	Exon 2	0.08	0.07	0.23	0.48	0.40
CPT2 (A)	FJ263683: g.41033G>A (p. Val397Ile)	Exon 4	0.71	0.66	0.51	0.61	0.60
MLYCD (A)	FJ263687: g.15996G>A (p. Gly388Asp)	Exon 5	0.09	0.71	0.00	0.08	0.53
FADS2 (A)	FJ263685: g.28505G>A	Intron 5	0.09	0.06	0.00	0.11	0.37
SCD1 (C)	AY487830: g.16663T>C	3' UTR	0.80	0.26	0.90	0.59	0.71
ACACB[2]	HT1: A-A-A-A	-	0.08	0.07	0.23	0.48	0.40
	HT2: A-A-A-G	-	0.09	0.42	0.00	0.04	0.00
	HT3: A-A-G-G	-	0.30	0.07	0.07	0.00	0.05
	HT4: A-G-A-G	-	0.53	0.42	0.70	0.38	0.11
	HT5: G-A-G-G	-	0.00	0.02	0.00	0.10	0.44

[1]Alleles in the parentheses correspond to alleles annotated as '1' in Table 3.10 and frequencies of these alleles are present here.

[2]Haplotypes were derived using four SNPs in *ACACB* with an order (g.20124A>G-g.25835A>G-g.25926G>A-g.26053 G>A).

Table 3.10 Results of association study between candidate genes and the lipid-related traits.

Gene	Sequence variants	Traits	Genotype / LSM ± SE (No. of animals)[1]			*P*-value[2]
			11	12	22	
ACACA	FJ263680: g.227756_ 227757ins280	n-3PUFA [%]	1.12 ± 0.06 (48)	1.28 ± 0.02 (258)	1.29 ± 0.02 (280)	0.04
ACACB	EU853705: g.20124A>G	n-3PUFA [%]	1.25 ± 0.03 (179)	1.32 ± 0.02 (274)	1.22 ± 0.04 (120)	0.02
	EU853705: g.25926G>A (p. Ser116Asn)	BTFM [mm]	29.43 ± 0.45 (148)	28.91 ± 0.31 (296)	30.26 ± 0.48 (140)	0.05
	EU853705: g.25926G>A (p. Ser116Asn)	SFA [%]	37.15 ± 0.12 (147)	36.86 ± 0.09 (292)	36.95 ± 0.12 (139)	0.07
	EU853705: g.25926G>A (p. Ser116Asn)	n-3PUFA [%]	1.25 ± 0.03 (148)	1.31 ± 0.02 (295)	1.24 ± 0.03 (140)	0.05
FADS2	FJ263685: g.28505G>A	IMF [%]	2.02 ± 0.08 (67)	2.01 ± 0.03 (279)	2.18 ± 0.04 (212)	0.02
	FJ263685: g.28505G>A	BTFW [mm]	45.17 ± 0.76 (67)	46.03 ± 0.33 (277)	44.33 ± 0.41 (210)	0.006
	FJ263685: g.28505G>A	BTFL [mm]	27.50 ± 0.78 (67)	26.88 ± 0.34 (279)	25.84 ± 0.41 (212)	0.09
	FJ263685: g.28505G>A	BTFS [mm]	43.69 ± 1.06 (67)	43.66 ± 0.46 (279)	41.41 ± 0.56 (212)	0.009
	FJ263685: g.28505G>A	ABFT [mm]	33.73 ± 0.65 (67)	34.27 ± 0.28 (279)	33.22 ± 0.34 (212)	0.06
SCD1	AY487830: g.16663T>C	MUFA [%]	48.06 ± 0.12 (264)	48.38 ± 0.11 (322)	48.37 ± 0.57 (11)	0.09

[1]Alleles designated as ‘1’ are presented in Table 3.9.

[2]Only results are shown with nominal *P*-values of < 0.1.

ACACA plays a critical role in the synthesis of fatty acids because its product (malonyl - CoA) is the two-carbon donor in the synthesis of saturated fatty acids catalyzed by fatty acid synthase and also in the chain elongation of saturated and unsaturated fatty acids to very long-chain fatty acids by different elongases (Leonard *et al*. 2004). Recently, *ACACA* has been tested as a positional and functional candidate for a QTL for fatty acid composition on SSC12 and shown to affect palmitoleic (C16:1 n-7), stearic (C18:0) and vaccenic (C18:1 n-7) fatty-acid concentrations in backfat (Munoz *et al*. 2007). Two synonymous SNPs analyzed by Munoz *et al*. (2007) were not identified in our study. However, we found an association of an insertion/deletion polymorphism (g.227756_ 227757ins280) involving a short interspersed nuclear element (SINE) in intron 44 of *ACACA* with the total n-3 PUFA in the intramuscular fat (Table 3.10). Hasler & Strub (2006) suggested potential involvement of SINE in gene regulation. Thus, it will be interesting to investigate whether the observed association results from altered gene expression.

Findings in several knockout experiments in mice have consistently demonstrated that ACACB is a key regulator of mitochondrial fat oxidation (Abu-Elheiga *et al*. 2001; Oh *et al*. 2005b; Choi *et al*. 2007). Mice lacking ACACB were characterized by continuous fatty acid oxidation and reduced fat storage, and also protected against diet-induced obesity and diabetes. Thus, *ACACB* represents an excellent candidate gene for lipid-related traits in pigs. We observed a suggestive association between the g.25926G>A(p. Ser116Asn) SNP and BFT at mid-back (Table 3.10). In addition, there was a tendency of this SNP to be associated with the SFA content in muscle (Table 3.10). Interestingly, a QTL with an effect on the SFA content in muscle (Sanchez *et al*. 2007) was found in the proximity of porcine *ACACB*, which was predicted to lie on SSC14 (Table 3.5). An association of SNPs: g.20124A>G and g.25926G>A (p. Ser116Asn) in *ACACB* with the total n-3 PUFA was found (Table 3.10). The Ser116Asn substitution has a benign effect on the protein structure and function (see above). Sequence comparison demonstrated that the g.20124A>G SNP is present in a region corresponding to a sterol regulatory element-binding protein binding element (SRE) in human *ACACB* promoter II (Oh *et al*. 2003), but this SRE is not conserved in the porcine putative promoter II (data not shown). Furthermore, a separate prediction revealed no additional regulatory site in the region containing the g.20124A>G SNP. Thus, it is unlikely that the g.20124A>G SNP alters *ACACB* expression. The associations observed in *ACACB* might be due to the linkage disequilibrium with the causal

mutations.

The ß-oxidation of long-chain fatty acids in mitochondria is a major source of energy, and the carnitine palmitoyltransferase II (CPT2) is involved in the transport of such fatty acids into the mitochondria. Consistent with its role, deficiency of CPT2 results in mitochondrial fatty acid oxidation disorders in humans (Bonnefont *et al*. 1999). Porcine *CPT2* was predicted to be located within a region on SSC6 (Table 3.5) where a QTL for average backfat thickness was found (Mohrmann *et al*. 2006). No association ($P \leq 0.05$) was detected between the g.41033G>A(p. Val397Ile) SNP in *CPT2* and the investigated lipid-related traits.

Malonyl Coenzyme carboxylase (MLYCD) catalyzes the degradation of malony-CoA to acetyl-CoA. The enzyme affects lipid partitioning because malony-CoA is the immediate precursor for *de novo* synthesis of fatty acids as well as a potent inhibitor of the CPT1 enzymes, thus a regulator of fatty acid oxidation. Patients deficient in MLYCD show a number of phenotypes reminiscent of mitochondrial fatty acid oxidation disorders (Wightman *et al*. 2003). Based on the location prediction (Table 3.5), *MLYCD* falls into a maternally expressed QTL interval for IMF on SSC6p (de Koning *et al*. 2000). The functional and positional evidences appear not to preclude *MLYCD* from being the candidate gene for this QTL. However, in humans or other species, imprinting has not been reported for this gene. No association ($P \leq 0.05$) between the g.15996G>A(p. Gly388Asp) SNP in *MLYCD* and IMF was found in the M × P family.

The Δ 5 and Δ 6 desaturases (FADS1 and FADS2, respectively) are the rate-limiting enzymes in mammalian synthesis of long-chain polyunsaturated fatty acids. Several studies in humans revealed that polymorphisms and haplotypes in the *FADS1-FADS2* or *FADS1-FADS2-FADS3* gene cluster show associations with levels of PUFA in serum phospholipids and in erythrocyte membranes (Schaeffer *et al*. 2006; Koletzko *et al*. 2008; Malerba *et al*. 2008; Rzehak *et al*. 2008). It has also been demonstrated that *FADS2* polymorphisms are associated with fatty acid profiles in the Japanese quail egg yolk, especially the n-6 and n-3 PUFAs (Khang *et al*. 2007). These findings strongly suggested that the *FADS* gene cluster is a promising candidate for the fatty acid composition in pigs. However, we found no association between the g.28505G>A SNP in *FADS2* and fatty acid composition. This initial analysis only used an intronic SNP, which was unlikely to be functional, and

therefore, was not enough to draw a final conclusion that the *FADS* genes should be dismissed as candidate genes for fatty acid composition in pigs. Interestingly, we observed an association of the g.28505G>A SNP with IMF and several backfat thickness measurements (Table 3.10). The involvement of *FADS2* in lipid metabolism seems to provide a plausible explanation for the associations. However, porcine *FADS2* was predicted to lie on SSC2p (Table 3.5), possibly 25 cM from the QTL for muscle growth and lipid deposition represented by the *IGF2* intron3-g.3072G>A causal mutation (Van Laere *et al*. 2003), and furthermore to be located in the intervals of two QTLs for fatness traits distinct from the former QTL (de Koning *et al*. 2000; Milan *et al*. 2002). Therefore, it cannot be excluded that the observed associations with fatness are due to linkage disequilibrium with the *IGF2* intron3-g.3072G>A mutation, or some unidentified causal ones, or both if the mentioned QTLs are segregating in our population. No QTL affecting IMF has been reported in the proximity of *FADS2* so far.

Stearoyl-CoA desaturase (SCD) is a crucial lipogenic enzyme necessary for the *de novo* biosynthesis of monounsaturated fatty acids in mammals. Mice deficient in *SCD1* display reduced body adiposity and increased insulin sensitivity, and are resistant to diet-induced weight gain (Ntambi *et al*. 2002), indicating its critical role in energy homeostasis. For this reason, Ren *et al*. (2004a) has tested it as a candidate for backfat traits in pigs. However, the analysis of a promoter SNP, not found in our population, showed the lack of its association with backfat thickness in a pure Duroc population. No association was found between the segregating g.16663T>C SNP with backfat thickness traits in our population, either. In order to investigate the genetic factors that affect fatty acid composition of beef and milk, bovine *SCD1* has been chosen as a promising candidate gene in several studies. Results revealed that polymorphisms in *SCD1* explain some of the observed variation of fatty acid composition in beef (Taniguchi *et al*. 2004) and milk (Kgwatalala *et al*. 2007; Mele *et al*. 2007; Milanesi *et al*. 2008; Schennink *et al*. 2008). The g.16663T>C SNP in porcine *SCD1* showed a tendency to be associated with the MUFA content in muscle (Table 3.10). In agreement with this finding, a suggestive QTL for the MUFA content in muscle (Sanchez *et al*. 2007) was identified in a region on SSC14 where porcine *SCD1* mapped (Ren *et al*. 2003). The g.16663T>C SNP is located in 3’ UTR, but no regulatory elements were found to contain the SNP (Ren *et al*. 2004b).

Discussion

In this study, a candidate gene approach was used to investigate the genetic basis of the lipid-related pork quality traits in pigs. Eleven candidate genes playing critical roles in lipid metabolism were selected because of strong a priori evidence for their involvement in the development of the traits of interest. Furthermore, most of these genes are located in relevant QTL regions obtained in the genome-wide scans through comparative mapping, making them potential positional candidates. However, at the beginning of this work, the absence of complete genomic sequences for most of the candidate genes rendered it impossible to test our candidate gene hypothesis. Thus, it was necessary to determine the genomic structure of these genes first. We took full advantage of the BAC end sequence information available at that time, and were able to quickly and efficiently identify specific BACs containing ten previously undescribed candidate genes. Although there are rough assignments for *ACACA*, *ACACB*, *CPT1A* and *CPT2* in the unfinished porcine genome sequence (http://pre.ensembl.org/Sus_scrofa/index.html) at present, our study generates genomic sequences of high quality for three *FADS* genes, *CPT1B*, *MLYCD* and *SCD5*, which are not available from the currently released porcine genome sequence. Furthermore, our study is the first to annotate these ten genes in pigs. We systematically re-sequenced the putative functional elements (promoter, 5' UTR, coding regions, intron-exon boundaries and 3' UTR). Finally, we presented a catalogue of sequence variants in these candidate genes. The variants identified in our study offer good chance to understand how these candidate genes affect the lipid-related traits in pigs.

It is not practical to genotype all the identified variants in each gene for association study due to the high cost involved. Therefore, the sequence variants need to be prioritized according to their functional significance. As presented in Table 3.7, the majority of the variants are located in the non-coding regions. Although they theoretically might lie in unknown regulatory elements, it is often difficult to assess their function on the basis of nucleotide sequence only (Tabor *et al*. 2002). It also holds true for synonymous SNPs, which are found to modulate the protein expression by altering the mRNA secondary structure (Nackley *et al*. 2006). Thus, our study attempted to give high priority to the non-synonymous SNPs due to their potential effects on protein function and structure. However, in some cases where no non-synonymous SNPs were identified (Table 3.7), variants in putative promoter, 3' UTR or introns were analyzed. Determining the linkage

disequilibrium between the variants in the candidate genes, not covered in our study, can also optimize the selection of variants for genotyping (Tabor *et al*. 2002).

In our preliminary association study, nine sequence variants in six candidate genes were analyzed using the M × P F_2 animals. We showed nominal evidence of association with the lipid-related traits for the variants found in *ACACA*, *ACACB* and *FADS2* ($P < 0.05$, Table 3.10). Of them, the intronic SNP in *FADS2* shows the strongest association ($P = 0.006$). However, none of these associations were significant after the Bonferroni correction of $P = 4.6 \times 10^{-4}$. The Bonferroni correction protects against false positive finding. This adjustment is highly conservative because it does not take into account correlation between traits and sequence variants. Thus, this increases the occurrence of the type II errors (false negatives). Even without performing the multiple testing correction, the observed associations should still be interpreted with caution. Because of considerable linkage disequilibrium (LD) existing in the F_2 population, it is often impossible to determine whether the observed effects are due to the candidate genes or their respective linked genes. To draw clear conclusions, our plan is to follow-up the significant or suggestive findings in the study (using an anti-conservative *P* value threshold of 0.1) in different pig populations to determine if these findings can be confirmed in them. The segregation of the variants nominally significant or suggestive for association with the lipid-related traits in the F_2 population has been studied in several pig breeds (Table 3.9). More extensive association studies are presently underway. Nonetheless, to obtain a thorough evaluation of the candidate genes listed in this study, much work remains to be done, e.g. investigating more variants and performing extensive haplotype analysis in different independent populations. If specific alleles or haplotypes in these candidate genes showing effects on the lipid-related traits in pigs could be identified and verified, it would contribute to the use of marker-assisted selection in breeding programmes.

Chapter 4

General discussion

General approach

A candidate gene approach was used in this study in order to identify the genes responsible for the economically important pork quality traits, especially the lipid-related traits (fatness, intramuscular fat content and fatty acid composition). First, a hypothesis was generated that the genes involved in lipid- and energy metabolism might affect the lipid-related pork quality traits. In the following step, selected candidate genes were characterized and screened for sequence variants. Finally, variants were investigated in different populations of pigs for association studies. The data presented in this study revealed some evidence for association between the candidate gene polymorphisms and the lipid-related traits that were investigated.

Selection of candidate genes

The choice of suitable candidate genes that may plausibly play a relevant role in the development process of a given trait is the first critical step in the candidate gene approach. The selection process can be considered an 'educated guess' about the genetic basis of the trait of interest (Kwon & Goate 2000). There are up to 30,000 protein-coding genes in the human genome. To limit the number of the genes for selection, several criteria have been used in many association studies (reviewed by Hattersley & McCarthy 2005). They include (1): the biology of a trait of interest; (2) knowledge from the animal models: the identification of genes influencing related traits in animal models offers candidate for testing in the target species; (3) prior association data; (4) positional information: genome-wide scans for linkage or association could indicate regions with a high probability of containing a causative gene; (5) functional candidates indicated by gene expression studies.

Each method mentioned above is not effective when used in isolation. For example, linkage analysis identifies a chromosomal region corresponding to a quantitative trait locus, which can be used for seeking candidate genes. However, it happens quite often that ten to hundreds of genes are harbored in the targeted chromosome region. Knowledge from the animal models provides clear functional links between gene dysfunction and whole body phenotype. However, the phenotypic similar trait of different species could have a quite different genetic architecture (Zhu & Zhao 2007). The available expression data are invaluable for identifying candidates for traits of specific interest. Nevertheless, there are often too many differentially expressed genes identified in the expression analysis. Further

validation of each gene for selection would be expensive and arduous. Random selection of a single gene or several genes would lead to a low probability of capturing the most relevant candidate genes. Therefore, a combination of at least two methods mentioned above is expected to be more effective in mining candidate genes.

The combination of QTL analysis and knowledge from the knockout mice has successfully identified diacylglycerol O-acyltransferase gene (*DGAT1*) for milk fat content in cattle (Grisart *et al*. 2002; Winter *et al*. 2002). The Lys232Ala substitution detected in bovine *DGAT1* was found to have a major effect on this trait. In this study, the selection of candidate genes was attempted based on a combined strategy. From a purely biological perspective, genes involved in lipid- and energy metabolism are strong candidates for the lipid-related traits (fatness, intramuscular fat content and fatty acid composition). Prior knowledge about the function of potentially contributing genes from other species (e.g. humans, mice, rats or cattle) was obtained through literature review. The potentially important genes were analyzed in relation to the QTL regions for the target traits identified in various genome-wide scans through comparative mapping using the Pig QTL database available at the website: http://www.animalgenome.org/QTLdb/pig.html. Based on the above information, twelve candidate genes encoding key enzymes or key regulators in lipid- and energy metabolism, therefore having potential contribution to the development of the lipid-related traits, were selected (Table 4.1). All but *CPT1B* and *SCD5* are located in the relevant QTL. The porcine clone map and comparative mapping allowed the assignment of *SCD5*, encoding the second SCD isoform in pigs, to a region on SSC8, where no QTL affecting lipid deposition and fatty acid composition have been reported so far. However, it should be noted that a very limited number of experiments have reported the identification of QTL affecting fatty acid composition in pigs. The chromosomal localization of porcine *CPT1B* could not be reliably predicted. The ongoing porcine genome sequencing project will provide an answer to this. Nevertheless, both genes are functional candidates.

Table 4.1 Twelve selected candidate genes in this thesis.

Function category	Gene symbol	Protein name	Knockout animals (-/-) / *Mutations in humans*	QTL identified in pigQTL database[a]	Association found in different species[b]
Fatty acid biosynthesis	*ACACA*	Acetyl-CoA carboxlase alpha	Early embryonic lethal (Abu-Elheiga *et al*. 2005)	C16:1 n-7, C18:0 and C18:1 n-7 in backfat	C16:1 n-7, C18:0 and C18:1 n-7 in backfat (in pigs)
Fatty acid oxidation (Regulators)	*ACACB*	Acetyl-CoA carboxlase beta	Lean with increased food intake (Abu-Elheiga *et al*. 2001)	SFA content in muscle	
	CPT1A	Carnitine palmitoyltransferase 1A (liver)	Embryonic lethal (Nyman *et al*. 2005)	Backfat thickness	Some obesity phenotypes (in humans)
	CPT1B	Carnitine palmitoyltransferase 1B (muscle)	Embryonic lethal (Ji *et al*. 2008)		Some obesity phenotypes (in humans)
	CPT2	Carnitine palmitoyltransferase II	*Fatty acid oxidation disorder (Bonnefont et al. 1999)*	Backfat thickness	
	MLYCD	Malonyl-CoA decarboxylase	*Fatty acid oxidation disorder (Wightman et al. 2003)*	IMF	
Fatty acid desaturation	*FADS1*	Δ5 desaturase	Abolishing PUFA synthesis (Stoffel *et al*. 2008)	Backfat thickness	Levels of PUFA (polymorphisms and haplotypes in human *FADS* gene cluster and polymorphisms in Japanese quail *FADS2*)
	FADS2	Δ6 desaturase		Backfat thickness	
	FADS3	Fatty acid desaturase 3		Backfat thickness	
	SCD1	Stearoyl-CoA desaturase 1 (Δ9)	Lean with increased food intake (Ntambi *et al*. 2002)	MUFA content in muscle	Fatty acid composition in milk and beef (in cattle)
	SCD5	Stearoyl-CoA desaturase 5 (Δ9)			
Energy sensor	*PRKAA2*	5'-AMP-activated protein kinase, catalytic alpha-2 subunit	Insulin resistance (Jorgensen *et al*. 2004)	Loin muscle area, IMF and backfat thickness	Insulin resistance and type 2 diabetes (in humans)

[a]http://www.animalgenome.org/QTLdb/pig.html.

[b]*ACACA* (Munoz *et al*. 2007), *CPT1A* and *CPT1B* (Robitaille *et al*. 2007), *FADS* (Schaeffer *et al*. 2006; Khang *et al*. 2007; Koletzko *et al*. 2008; Malerba *et al*. 2008; Rzehak *et al*. 2008), *PRKAA2* (Horikoshi *et al*. 2006).

Genomic characterization

Genomic characterization of genes is a critical step toward their functional analysis. When this work started in June 2005, an extensive screening for porcine sequences was made for the selected candidate genes. Human mRNA reference sequence of each candidate gene was used as a query sequence to 'BLAST' separately against five databases in GenBank: Nucleotide Collection (nr/nt); Non-human, Non-mouse ESTs (est-others); Genomic survey sequences (gss); High throughout genomic sequences (HTGS) and Reference mRNA sequences (refseq_rna). Although the porcine genome sequencing project has been launched, BLAST results obtained at that time showed a collection of porcine ESTs and genomic survey sequences for most of the selected candidate genes. The identified genomic survey sequences were small and separate fragments (<1 kb) and did not cover the entire gene. Hence, in order to get genomic sequences for the selected candidate genes for further analysis, it was necessary to perform gene cloning. Fortunately, a large number of BAC end sequences (BES) from different porcine BAC libraries were released from the Porcine Genome Physical Mapping Project at that time, which allowed us to develop *in silico* BAC library screening approach. Thus, it was possible to pick potential positive BACs through analysis of the available data on the internet. Using the approach, it was successful to identify BACs for the genes analyzed in the study. Compared to the traditional hybridization-based screening approach, which was generally time-consuming and labour-intensive, the approach used was a quick and efficient method, greatly facilitating the isolation of target genes. Each consensus base in the genomic contigs derived from BAC shotgun sequencing had approximately 4-15 reading characters, which indicated high quality of the sequences. Based on these derived sequences, genomic structures of the candidate genes were determined by similarity-based gene prediction and subsequent manual curation.

One contribution of manual annotation to genome analysis is the careful annotation of alternative variants (Ashurst & Collins 2003). Analyzing a large number of available EST/cDNA sequences for porcine *CPT1A* unveiled possible alternative splicing events in both 5' and 3' end of this gene. For porcine genes (*CPT1B*, *FADS1*, *FADS2*, *FADS3*, *MLYCD*, *PRKAA2* and *SCD5*), there was no possible alternative splicing observed in the 5' and 3' end based on their respective 5' and 3' EST/cDNA sequences available so far. For porcine *ACACA* and *ACACB* genes, it was a challenge to annotate the 5' and 3' end given

the facts that very few or no ESTs were available for *ACACA* and *ACACB* respectively and that very complicated organization in 5' end has been reported for both genes in humans. However, it has been generally observed in this study that gene structures are conserved between pigs and humans, and there is no reason to think that conservation is not maintained between spliced variants. Therefore, the putative extents of exons in the 5' end and 3' end of porcine *ACACA* and *ACACB* were primarily defined by homology with the corresponding exons in human *ACACA* and *ACACB*.

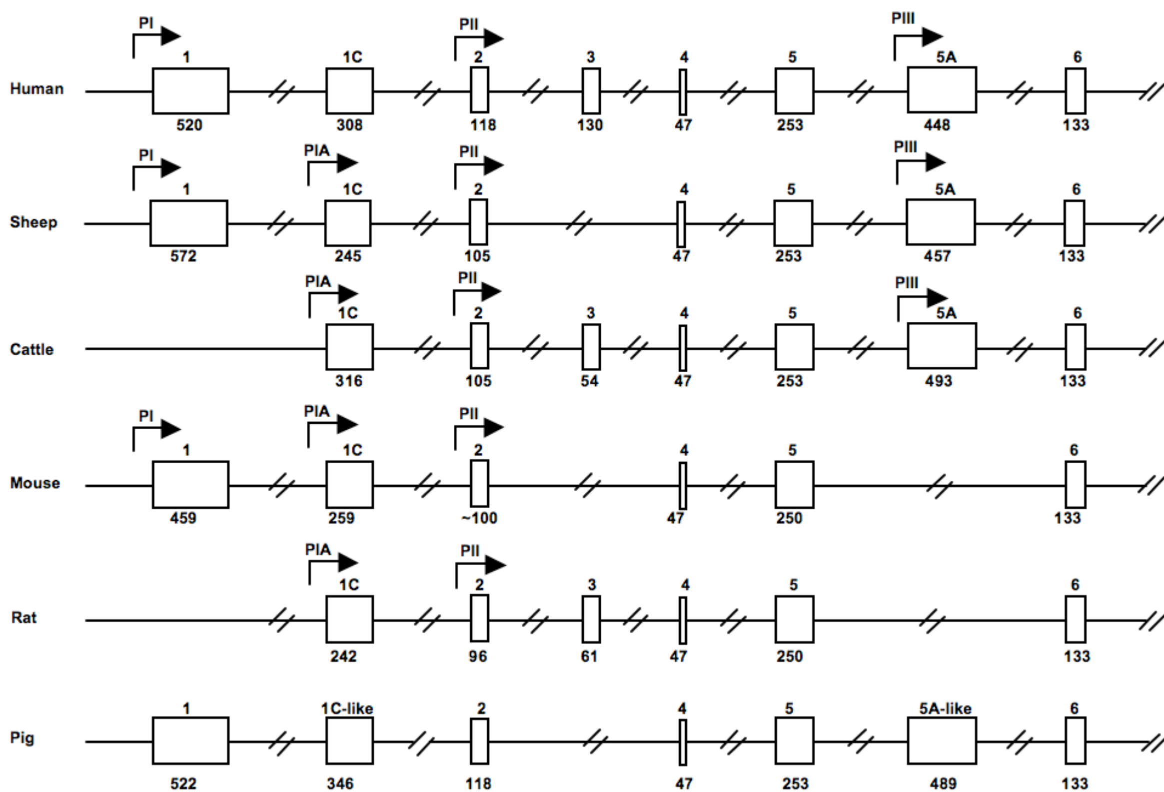

Figure 4.1 The organization of 5' region of *ACACA* in mammals. The promoter numbering is based on suggestions made by Travers *et al*. (2005) and Barber *et al*. (2005). Genomic organization of human promoters and exons was indicated by Mao *et al*. (2003). Only the common exons are displayed here. In sheep, PIA and PII, and the exons downstream of them are organized in a structure suggested by Travers & Barber (2001). The distribution of bovine promoters and exons was shown in Mao *et al*. (2001) and Mao & Seyfert (2002). The organization of 5' region of rat *ACACA* was determined by Luo *et al* (1989). The mouse *ACACA* structure in the 5' region was suggested by Travers *et al*. (2003). The location of PI and exon 1 in mice and sheep was defined by Travers *et al*. (2005). In pigs, exon 1C-like and exon 5A-like were defined by the homology with the corresponding exons in humans.

However, such annotation based on the human information does not necessarily apply to porcine genes. This can be observed from the analysis of the organization of 5' region of *ACACA* in several investigated mammalian species, namely rats, mice, humans, cattle and sheep (Fig. 4.1). The presence of four promoters (PI, PIA, PII and PIII) has been described in the mammalian *ACACA* (reviewed by Barber *et al*. 2005). These promoters seem to have a species-specific expression pattern (Fig. 4.1). In contrast to humans and ruminants, rodents express no transcripts containing exon 5A as the leader exon although regions homologous to human exon 5A exist in the rodent *ACACA* genes. Hence, there is no corresponding functional PIII in the rodents. The PIA (previously annotated as PI) in the rodents (Luo *et al*. 1989) and ruminants (Mao *et al*. 2001; Travers *et al*. 2001) initiates the transcription starting at exon 1C (previously designated as exon 1). However, although human exon 1C exists and its upstream sequence shows homology with the PIA in the rodents and ruminants, no transcript containing exon 1C as a leader exon was detected, indicating that the region upstream of exon 1C may not function as a promoter in humans (Mao *et al*. 2003). Therefore, experimental work is required to confirm or refine the predictions for the porcine *ACACA* and *ACACB*. For the porcine *ACACA*, existence of exon 1 has been validated by 5' RACE analysis. In the case of the porcine *ACACB*, RT-PCR was only performed in the study to confirm the existence of exon 1a and exon 1b (two alternatively spliced non-coding exons, Fig. 3.1) in the 5' end. Further analysis is needed to validate the existence of exon 2a.

It is worth mentioning that the alternative splicing event may also be detected in the middle of a gene. For example, alternative splicing of exon 32 (Fig. 3.1, 24 bp) of *ACACA* has been consistently reported in humans, rats and sheep (reviewed by Barber *et al*. 2005). The presence/absence of exon 32 in transcripts results in inclusion/exclusion of an 8-aa stretch in proteins. Because rat Ser1200 residue (Ser1201 in porcine ACACA, Fig. 3.2) resides in the 8-aa region, the alternative splicing of exon 32 finally influences the phosphorylation of Ser1200, which involves the regulation of the ACACA enzyme. Recently, Gallardo *et al*. (2008) also described the similar alternative splicing event in the porcine *ACACA*. However, the annotation based on the available porcine EST/cDNA sequences failed to detect this alternative splicing, indicating that EST/cDNA coverage is probably a major influencing factor to whether alternative splicing is detected during the manual annotation (Ashurst & Collins 2003; Pan *et al*. 2008).

The manual annotation can also help predict promoters and polyA sites if full-length cDNA sequences are available. This was not possible in our case since few 5' and 3' EST/cDNA sequences could be used in the annotation for most of the candidate genes. On the other hand, most of the 5' EST/cDNA sequences submitted to the nucleotide database are often incomplete. Thus, in this study the 5' end of the genes analyzed was determined by the EST/cDNA sequence with the longest 5' UTR if there were 5' EST/cDNA sequences available. If not available, the putative extent of the 5' end was defined based on homology with human genes. The same rule was used in determining the 3' end of the porcine genes.

Sequence variants

One goal of this study was to identify sequence variants in candidate genes. The putative functional elements (5' end, coding regions, intron-exon boundaries and 3' end) were targeted for re-sequencing. In total, 92.5 kb in twelve selected genes were re-sequenced and 392 sequence variants were identified including 383 SNPs, seven insertion-deletion polymorphisms and two microsatellites.

SNPs, the most prevalent sequence variations, are consequences of either transition or transversion events. In the present work, transitions A/G and C/T are over-represented with 38.9% and 38.3% of the total 383 SNPs, respectively, whereas four transversion classes (A/C, A/T, C/G and G/T) together account for 22.7%. Thus, transitions occurred 3.4 times more frequently than transversions. The transition over transversion ratio falls within a range of 1.7 to 4 observed in the SNP studies in mammals and birds (Vignal *et al*. 2002). The transition bias is probably partly related to 5-methylcytosine deamination reactions that are known to occur frequently, particularly at CpG dinucleotides (Holliday & Grigg, 1993).

With these available sequence variants, the question arises: Which sequence variants are more important and should be further investigated? Tabor *et al*. (2002) suggested that information about the location of the sequence variants in a gene could be used to prioritize polymorphisms. Noticeably, the sequence variants that are located in the coding regions and have effects on protein function and structure should be given the highest priority for genotyping in association studies. In this study, eleven SNPs, which cause amino acid exchanges, were identified. Some of them are located in the important functional or structural domains. For example, the Met2Val and Asp13Gly substitutions in CPT2

occurred in the porcine N-terminal region homologous to rat and human CPT2 mitochondrial targeting sequences (Fig 3.2). The Gly203Ser substitution was found in a putative inner mitochondrial associated motif and residue Gly203 was completely conserved in several mammals (Fig 3.2, Table 3.8). Two substitutions (Gln328Arg and Asn537Ser) in porcine CPT1A, one (Asp253Asn) in SCD5 and one (Gly388Asp) in MLYCD occurred in the regions related to their respective protein function (Fig 3.2). Nevertheless, none of these substitutions appear to have damaging or deleterious effect on protein function or structure based on the prediction using the online tool PolyPhen (Ramensky *et al*. 2002). Two amino acid exchanges in porcine ACACB lie between the very N-terminal hydrophobic region and the BC domain (Fig. 3.2). As predicted by the PolyPhen, it is most unlikely that they could have impact on the function and structure of the protein. This could indicate that non-synonymous sequence variants identified in this study are not of great interest for association studies.

The second class of sequence variants that gains increasing attention is the regulatory variant group. These variants could include any variant that affects regulation of gene expression without changing an amino acid of the protein (Crawford *et al*. 2005). It is generally assumed that changes in the promoter could affect transcription factor binding or changes in the UTR sequence could affect mRNA stability or translation. Hence, variants that are identified in promoters or untranslated regions (UTR) of the gene are likely candidates for variants that could affect gene expression (Crawford *et al*. 2005). A total of 45 sequence variants were found in 5' end in this study, and 33 in 3' end. The 5' end sequences in *ACACA* and *CPT1A* were not screened for sequence variants in this thesis, which would be worth investigating in the future. Although it is generally difficult to predict the effect of a variant in 5' or 3' end on gene expression on the basis of the DNA sequence only, Tabor *et al*. (2002) suggested that if a sequence variant is found in a highly conserved element based on comparison of related gene sequences, it is likely that this variant will have functional significance. Therefore, such a sequence variant should be given a high priority for further investigation.

In the present study, 74 SNPs in coding regions without changing amino acid residues and 229 polymorphisms in introns were identified. Although such sequence variants may possibly have functional significance, predicting their effect is a demanding task. Nevertheless, it was suggested that synonymous variants should be given higher priority for

genotyping than the variants lying deep within introns since synonymous SNPs have potential effect on mRNA stability (Capon *et al*. 2004; Tabor *et al*. 2002).

Association results

Association of the *PRKAA2* sequence variants with lean- and fat deposition

PRKAA2 was chosen as a functional candidate for lean- and fat deposition because of the critical role of AMPK in the regulation of energy homeostasis. No prior association analysis in the porcine *PRKAA2* gene was available. The candidate gene analysis presented in this study revealed association of the porcine *PRKAA2* genotypes with loin muscle area in F_2 population of the M × P family. In an attempt to extend the result obtained in the F_2 population, the association between the *PRKAA2* genotypes and traits of interest was studied in two commercial pig populations. However, the observed association in the F_2 population was not confirmed in the commercial pigs. The inconsistent effects observed across the analyzed populations indicate that the investigated sequence variants are unlikely to be causal, but in linkage disequilibrium with the putative causal mutation. The inconsistency also indicates that different linkage disequilibrium might exist among these pig populations. This is likely since the nature and extent of linkage disequilibrium differ from population to population. For example, F_2 populations that involve crosses are expected to have extensive and long-range linkage disequilibrium while purebreds are expected to have the least. Because the association observed in the F_2 population was detected after adjustment of the *RYR1* mutation on SSC6q11-q12 (https://www-lgc.toulouse.inra.fr/pig/cyto/gene/chromo/SSCG6.htm), this seemed to suggest that in addition to the *RYR1* locus there could be the different locus (loci) on SSC6 affecting muscle growth in pigs. In agreement with this, QTL affecting loin muscle area were reported to be close to marker SW322 on SSC6q31-q35 (Ovilo *et al*. 2002b; Edwards *et al*. 2008). This marker is closely linked to the porcine *PRKAA2* gene based on the RH mapping result.

It is very interesting to note that leptin receptor gene (*LEPR*) is functionally and positionally related to *PRKAA2*. AMPK functions as an important downstream mediator of the effects of leptin on energy balance (Minokoshi *et al*. 2002; Minokoshi *et al*. 2004). In humans, both *PRKAA2* and *LEPR* are located on chromosome 1p31, with a distance of 8.5 Mb between them. In pigs, both genes map to SSC6q (*PRKAA2*: 6q31-q35 in this study,

LEPR: 6q33-q35 in Ernst *et al*. 1997) although the exact distance between them cannot be determined yet due to the incomplete sequence on SSC6. Based on the functional and positional evidence, both genes can be considered promising candidates for the above-mentioned QTL for loin muscle area. A candidate gene analysis in pigs indicated that haplotypes of porcine *LEPR* are responsible for some of QTL effects for backfat thickness measurements on SSC6 (Ovilo *et al*. 2005). Association of variants in porcine *LEPR* with loin muscle area was not observed. In the present work, association of the *PRKAA2* variants was found with loin muscle area, but no association was found with fatness traits. On the other hand, this study could not detect a genetic variant in *PRKAA2* that is likely to cause the observed association (all 25 polymorphisms identified are most likely non-functional mutations). Nevertheless, it appears to confirm the reported QTL for loin muscle area on SSC6q. Therefore, evaluation of more candidate genes in the QTL region will help identify the causative mutation for loin muscle area on SSC6q.

Association of sequence variants in genes relevant to lipid metabolism with lipid deposition and fatty acid composition

The critical roles of eleven genes (*ACACA*, *ACACB*, *CPT1A*, *CPT1B*, *CPT2*, *FADS1*, *FADS2*, *FADS3*, *MLYCD*, *SCD1* and *SCD5*) encoding key enzymes or key regulators in lipid metabolism encouraged us to investigate them as promising candidates for the lipid-related traits in pigs. In a preliminary association study, only nine sequence variants in six genes (*ACACA*, *ACACB*, *CPT2*, *FADS2*, *MLYCD* and *SCD1*) were selected for analysis in the F_2 population of the M × P family on the basis of their potential functional significance or variant allele frequency. Five of these variants (*ACACA*-FJ263680: g.227756_ 227757ins280), *ACACB*-EU853705: g.20124A>G and EU853705: g.25926G>A (p. Ser116Asn), *FADS2*-FJ263685: g.28505G>A and *SCD1*-AY487830: g.16663T>C) were found to be associated with the lipid-related traits (lipid deposition or fatty acid composition in muscle). Not much work has been carried out in pigs to investigate the genetic basis of fatty acid composition in muscle. Sanchez *et al*. (2007) presented the first QTL analysis of fatty acid composition measured in pig meat. No other QTL studies or candidate gene analyses are available to date. This study reports association of variants in *ACACA* (FJ263680: g.227756_ 227757ins280), *ACACB* (EU853705: g.20124A>G and EU853705: g.25926G>A (p. Ser116Asn)) and *SCD1* (AY487830: g.16663T>C) with percentages of different fatty acids (e.g. n-3 PUFA, SFA or MUFA) in the intramuscular

fat. All the associated variants seem to have no functional significance and the associations are likely due to the linkage disequilibrium with the causal mutations. Nevertheless, this study hopes to facilitate further studies and contribute to further understanding of the genetic background of fatty acid composition in muscle. The intronic SNP in *FADS2* (FJ263685: g.28505G>A) was found to be associated with several backfat thickness measurements. Porcine *FADS2* was predicted to lie on SSC2p where a large number of QTL affecting fatness traits have been reported (see PigQTL database). The role of *FADS2* in lipid metabolism does not exclude this gene from being a candidate gene for backfat thickness. Hence, it is worth investigating this gene further in order to clarify its effect. The *CPT1A* gene, which is also located on SSC2p, has not been investigated in the preliminary association study. This gene can also be considered as another promising candidate for the backfat thickness QTL on SSC2p on the basis of its critical role in regulation of fatty acid oxidation.

The preliminary association analysis was performed with one or few sequence variants in each of the six genes (*ACACA*, *ACACB*, *CPT2*, *FADS2*, *MLYCD* and *SCD1*) using an F_2 population where considerable linkage disequilibrium was expected. Although sequence variants have been identified in the remaining five genes (*CPT1A*, *CPT1B*, *FADS1*, *FADS3* and *SCD5*), an association study has not been performed in them. For these reasons, a final conclusion could not be drawn about the effect of the eleven selected candidate genes. Further evaluation of these candidate genes is currently underway.

Appraisal of the candidate gene approach

In pigs, two approaches have been used for genetic dissection of complex quantitative traits: genome-wide linkage mapping and candidate gene approach. Linkage mapping relies on the anonymous markers evenly spaced throughout the genome, and prior knowledge of the physiology or biology underlying a given trait is not needed. A drawback of this approach is its high cost connected with the maintenance of experimental populations and genotyping a large number of markers. Although numerous QTL affecting complex quantitative traits have been detected using this approach, there are still great challenges in unraveling the genes that underlie them due to the imprecise chromosomal locations of QTL and the inability to distinguish the mutations underlying QTL with mild phenotypic effect (most QTL identified belong to this type) from neutral polymorphisms (Andersson &

Georges 2004). Therefore, as an alternative to the genome-wide linkage mapping approach, the candidate gene approach has also been widely used to identify the genetic basis for complex quantitative traits. A quick search through PubMed should be enough to convince anyone of this fact.

The candidate gene approach focuses on genes that are selected because of a priori hypothesis about their relevant role in the development process of a given trait. This method takes advantage of the biological understanding of genes and proteins that are likely to be involved in the trait of interest. Moreover, the candidate gene approach has broad applicability. Theoretically, any population, in which phenotyping can be done and genetic variations exist, can be used in an association study of candidate genes, e.g. purebreds, selection lines, commercial lines or F_2 populations. Lastly, the candidate gene approach is relatively economical and easy to use by smaller research labs or individuals interested in specific gene identification. Nevertheless, candidate gene studies have been criticized for non-replication and lack of thoroughness (reviewed by Tabor *et al*. 2002). The lack of reproducibility seen with candidate-gene studies can be partly attributed to variations in study designs such as different study populations used and different definition of the phenotypes. Another possible explanation for non-replication across candidate-gene studies relates to the selection of polymorphisms that are not likely to be causal. The *PRKAA2* candidate gene analysis presented in this study gives an example of non-replication. Because it was not possible to identify a potential functional mutation in *PRKAA2*, sequence variants were genotyped in order to capture association based on linkage disequilibrium with the causal mutation. Given the possibility of different linkage disequilibrium existing in different investigated populations, detecting the inconsistency is anticipated. However, the non-replication should not discourage anyone who wants to use this approach. In contrast, it signifies that both the study design and the interpretation of results have to be done with caution (Tabor *et al*. 2002; Hattersley & McCarthy 2005).

A ‘good’ association means the identification of a gene with a relevant role in the development of a given trait as well as the identification of functional sequence variants in this gene involved in regulating gene expression or changing gene product function. Thus, the successful prediction of functional candidate genes and functional sequence variants in them are crucial to the ‘good’ application of the candidate gene approach. However, it has been argued that current knowledge might be insufficient to make such prediction. For this

reason, candidate gene approach could have limited ability to include all possible causative genes and sequence variants. Fortunately, with the near-complete genome sequence and the effort in developing high-throughput SNP genotyping assays, the extension of the candidate gene approach to the whole genome-wide association approach, which does not need a prior hypothesis, is expected in pigs. Such an extension will help provide a comprehensive understanding of the genetic basis of many complex traits in pigs. The breakthrough in sequencing technology e.g. next-generation sequencing technologies currently available from 454 Life Sciences, Illumina and Applied Biosystems (Mardis 2008), enables the rapid and affordable whole genome sequencing. The more comprehensive approach toward understanding the complex traits in pigs in the future will be the complete genome sequencing approach (Hirschhorn & Daly 2005; Sellner *et al*. 2007).

Summary

Fatness and intramuscular fat content are the economically important quality traits because consumers desire both leanness and palatability in pork. Fatty acid composition has become another important quality trait in the pork industry due to its influence on human health. As quantitative traits, fatness, intramuscular fat content and fatty acid composition are all determined by both genetic and environmental factors. The aim of this study was to investigate the genetic basis of these lipid-related traits using the candidate gene approach. Twelve genes encoding key enzymes or key regulators involved in lipid- and energy metabolism, which therefore have potential contribution to the development of these lipid-related traits, were selected for analysis.

The twelve candidates include *PRKAA2* involved in regulation of energy balance and eleven genes involved in different lipid metabolic pathways: fatty acid *de novo* biosynthesis (*ACACA*), fatty acid degradation (*ACACB, CPT1A*, *CPT1B*, *CPT2* and *MLYCD*) and fatty acid desaturation (*FADS1*, *FADS2*, *FADS3*, *SCD1* and *SCD5*). Genomic organization has not been previously described for eleven of these genes in pigs. An *in silico* BAC library screening approach based on publicly available porcine BAC end sequences and human genome information was used to identify 13 BACs for the eleven undescribed genes. Gene structures were elucidated with BAC shotgun sequencing and semi-automatic annotation. Putative functional elements (5' end, coding regions, intron-exon boundaries and 3' end) were targeted for re-sequencing. In total, 92.5 kb in the twelve selected genes were re-sequenced and 392 sequence variants were identified including 383 SNPs, seven insertion-deletion polymorphisms and two microsatellites. Eleven of these SNPs caused amino acid substitutions.

Re-sequencing revealed 25 polymorphisms in *PRKAA2*. None of them caused amino acid exchanges. Haplotype construction based on the 25 sequence variants revealed four haplotypes in the parental generation of a Mangalitsa × Piétrain intercross. Two tag SNPs that could distinguish between three frequent haplotypes, were chosen for genotyping and were tested for association with traits of interest (muscle development and fat deposition) in the Mangalitsa × Piétrain F_2 cross (n = 589). Single SNP and haplotype analyses only revealed significant associations between the *PRKAA2* genotypes and loin muscle area. To overcome limitations due to linkage disequilibrium within the F_2 population, association

analyses using the two SNPs were extended to two commercial pig populations: Piétrain (n = 1173) and German Landrace (n = 536). However, the findings observed in the F_2 population could not be confirmed in these commercial populations. Thus, the two analyzed SNPs were unlikely causal and the observed associations could be due to linkage disequilibrium (LD) with the causal mutation. *PRKAA2* was physically mapped to porcine chromosome 6q31-35 using a radiation hybrid cell panel. Interestingly, a QTL affecting loin muscle area was found in the region where *PRKAA2* maps in some studies. However, this study could not detect a genetic variant in *PRKAA2* that was likely to cause the observed associations.

Re-sequencing also revealed sequence variants in all the eleven genes relevant to lipid metabolism. Nine sequence variants in six genes (*ACACA*, *ACACB*, *CPT2*, *MLYCD*, *FADS2* and *SCD1*) were genotyped in 580 pigs of the Mangalitsa × Piétrain F_2 population and association analyses with fatness, intramuscular fat content and fatty acid composition were performed. Four of these variants showed significant associations with the lipid-related traits. An intronic 280bp-SINE insertion/deletion polymorphism in *ACACA* was found to be associated with n-3 polyunsaturated fatty acid content (n-3 PUFA) in the *longissimus dorsi* muscle. An association with n-3 PUFA was also found for a promoter SNP and a non-synonymous SNP in *ACACB*. Furthermore, an association between the non-synonymous variant in *ACACB* and backfat thickness at mid-back was detected. An intronic SNP in *FADS2* showed an association with IMF and several backfat thickness measurements. Although several sequence variants were found to be associated with the lipid-related traits, conclusions could not be drawn that these variants are responsible for the observed effects. Significant results might also arise from the LD with the causal mutations, especially in the F_2 population where considerable LD exists. In this study, only preliminary association analyses between several of the selected candidate genes and the lipid-related traits were performed. The systematically identified variants in the eleven candidates genes present a good opportunity to understand how these genes affect the lipid-related traits in pigs. Therefore, further research (e.g. extensive association analyses of sequence variants in each candidate gene in different independent populations) remains to be done. Such further analyses will help provide a thorough evaluation of these candidate genes, hence contributing to a better understanding of the genetic background of the lipid-related traits.

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Abbreviations

A	adenine
ABI	Applied Biosystems
ACACA	acetyl-Coenzyme A carboxylase alpha gene
ACACB	acetyl-Coenzyme A carboxylase beta gene
AMPK	AMP-activated protein kinase
BAC	bacterial artificial chromosome
BES	BAC end sequences
BFT	backfat thickness
BLAST	basic local alignment search tool
bp	base pair
C	cytosine
cDNA	complementary deoxyribonucleic acid
cM	centi Morgan
CPT1A	carnitine palmitoyltransferase 1A (liver) gene
CPT1B	carnitine palmitoyltransferase 1B (muscle) gene
CPT1C	carnitine palmitoyltransferase 1C gene
CPT2	carnitine palmitoyltransferase II gene
DE	German Large White
DFCI	Dana-Farber Cancer Institute
DL	German Landrace
DNA	deoxyribonucleic acid
DU	Duroc
EST	expressed sequence tag
FADS1	delta-5 desaturase gene
FADS2	delta-6 desaturase gene
FADS3	fatty acid desaturase 3 gene
FASN	fatty acid synthase gene
G	guanine
HSA	human chromosome
HT	haplotype
HUFA	highly-unsaturated fatty acid
IMF	intramuscular fat content

indel	Insertion Deletion polymorphism
kb	kilo base pairs
LD	Linkage Disequilibrium
LMA	loin muscle area
M × P	a Mangalitsa × Piétrain intercross
Mb	Mega base pairs
MGB	minor groove binder
MLYCD	malonyl-Coenzyme A decarboxylase gene
mRNA	messenger ribonucleic acid
MUFA	monounsaturated fatty acid
N	A, C, G, T, U
n-3 PUFA	Omega-3 polyunsaturated fatty acid
n-6 PUFA	Omega-6 polyunsaturated fatty acid
NCBI	National Center for Biotechnology Information
PCR	polymerase chain reaction
PI	Piétrain
PRKAA1	AMP-activated protein kinase alpha 1 catalytic subunit gene
PRKAA2	AMP-activated protein kinase alpha 2 catalytic subunit gene
PRKAB1	AMP-activated protein kinase beta 1 non-catalytic subunit gene
PRKAB2	AMP-activated protein kinase beta 2 non-catalytic subunit gene
PRKAG1	AMP-activated protein kinase gamma 1 non-catalytic subunit gene
PRKAG2	AMP-activated protein kinase gamma 2 non-catalytic subunit gene
PRKAG3	AMP-activated protein kinase gamma 3 non-catalytic subunit gene
PUFA	polyunsaturated fatty acid
QTL	quantitative trait locus / loci
RACE	Rapid Amplification of cDNA ends
RFLP	restriction fragment length polymorphism
RH	radiation hybrid
RNA	ribonucleic acid
RT-PCR	reverse transcription PCR
RYR1	ryanodine receptor 1 (skeletal) gene, calcium release channel gene
SCD1	stearoyl-Coenzyme A desaturase 1
SCD5	stearoyl-Coenzyme A desaturase 5

SFA	saturated fatty acid
SINE	short interspersed nuclear element
SNP	single nucleotide polymorphism
SSC	porcine chromosome
T	thymine
UTR	untranslated region
ZDS	Zen-tralverband der Deutschen Schweineproduktion e.V

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Appendices

Table 1 Exon/intron organization of the porcine *ACACA* gene.

Exon	Size (bp)	Acc. No. FJ263680	3' splice acceptor	5' splice donor	Intron	Size (bp)
1	522	2865-3386		GGCTAG***gt***atgt	1	44996
1C-like	346	48383-48728		TCTCAG***gt***acag	1C-like	>4461
2	118	53290-53407		CTTGAG***gt***gagg	2	23556
3	?	?		?		
4	47	76964-77010	cttt***ag***TTCTTT	TAAGAG***gt***cagt	4	3837
5	253	80848-81100[a]	ccat***ag***CTCTGA	CATGAG***gt***aagt	5	19096
5A-like	489	100197-100685		TCAAAG***gt***aagt	5A-like	4938
6	133	105624-105756	ttgt***ag***GTCCAG	GAGAAG***gt***aagt	6	1311
7	139	107068-107206	tccc***ag***GTTCTC	ACGCTG***gt***gagt	7	5054
8	110	112259-112368	tttc***ag***AATACA	GTGCAA***gt***aaga	8	1075
9	82	113444-113525	ttgt***ag***GCAGTA	TCATGG***gt***aaga	9	1742
10	99	115268-115366	tttt***ag***GTCCTC	GCAGTG***gt***aaga	10	1870
11	107	117237-117343	ttgc***ag***GTCTTC	CTGAAG***gt***aggt	11	3647
12	111	121012-121122	ttga***ag***GCAGCA	AGACAG***gt***agag	12	5995
13	210	127115-127324	atat***ag***GTTCAA	GAACAG***gt***acat	13	3942
14	171	131267-131437	tttg***ag***TGTGCG	CTCCAG***gt***atgt	14	986
15	162	132424-132585	tttc***ag***ATTGCC	GATGAG***gt***aacc	15	559
16	164	133145-133308	caac***ag***GGTTTC	AATTTC***gt***gagt	16	2829
17	151	136138-136288	tccc***ag***AAACAT	GTACAG***gt***gtgt	17	1313
18	104	137602-137705	tttc***ag***GCAGAG	GGAGAG***gt***aggc	18	2782
19	82	140488-140569	tttc***ag***GGGTCA	CTGAAG***gt***atgc	19	438
20	146	141008-141153	ctac***ag***GTGACT	GGATAG***gt***gagt	20	1536
21	151	142688-142838	tcct***ag***ATATCG	ATCGAG***gt***cagg	21	1054
22	135	143893-144027	ttat***ag***GTGATG	CAGCAG***gt***gagg	22	342
23	147	144370-144516	tgac***ag***GCTGAG	AGTAGG***gt***atga	23	774
24	189	145291-145479	cctc***ag***GTAAAA	CAGCAG***gt***attt	24	1578
25	101	147058-147158	cttt***ag***ATTGCC	GCAGAG***gt***agtt	25	788
26	89	147947-148035	gccc***ag***GTACCG	AGAACG***gt***aagc	26	5529
27	125	153563-153687	ttgc***ag***GTCACT	CTTATC***gt***gagt	27	>7419
28	114	161207-161320	actt***ag***GATCAG	CGCCAG***gt***gagg	28	1224
29	114	162545-162658	ctct***ag***GTTCTT	CTGCAG***gt***acac	29	1007
30	90	163666-163755	ttcc***ag***AAACTC	CTGGAG***gt***aggc	30	1828
31	119	165584-165702	aacc***ag***GTGTAT	AAACAG***gt***gagt	31	10644
32	24	176342-176365	ttac***ag***AGGGAA	AAACAG***gt***accg	32	2627
33	144	178992-179135	attt***ag***AATGTC	TGTCAG***gt***gagg	33	769
34	97	179905-180001	tttc***ag***GATCTT	GACAAG***gt***atag	34	910
35	108	180912-181019	ttac***ag***GTCCCC	CAAAAT***gt***aagt	35	5128
36	57	186148-186204	ttgc***ag***AAAGCT	CAAAAG***gt***attg	36	226
37	45	186431-186475	tgaa***ag***GATTTC	TTTCAT***gt***aagt	37	7171
38	42	193643-193684	acgc***ag***AGAGAA	GATAAG***gt***aagg	38	1045
39	216	194730-194945	gttc***ag***TTTGAG	ACCAAG***gt***gggt	39	1808
40	156	196754-196909	gtgt***ag***GAAGCC	TCAAAG***gt***acat	40	2305
41	204	199215-199418	tgtc***ag***ATTGAG	GCACAG***gt***acag	41	5453
42	156	204897-205052	ctgc***ag***ATCATG	CGGCAG***gt***aaag	42	2511
43	147	207560-207706	cttt***ag***TCCCTG	AATGAG***gc***aagt	43	>15338
44	270	223145-223414	ttgc***ag***ATTGGC	TACAAG***gt***acag	44	4959
45	98	228374-228471	tcac***ag***GGATAC	ATCCAG***gt***aggt	45	4747
46	121	233219-233339	cact***ag***GTACAA	AGCCTG***gt***aaat	46	573
47	111	233913-234023	ttta***ag***GTTACC	AACAAA***gt***aagt	47	17966
48	144	251990-252133	ctcc***ag***GTTCTC	CCCAAG***gt***gagc	48	1280
49	121	253414-253534	tttt***ag***AGCGTA	ACCCAA***gt***atgt	49	3325
50	97	256860-256956	ccgt***ag***CCCAGA	AGCTAG***gt***aagt	50	3358
51	97	260315-260411	tatc***ag***GCTAGG	GCCAAG***gt***aggt	51	899
52	136	261311-261446	caat***ag***ATAATC	TGAAAG***gt***gatt	52	7725
53	178	269172-269349	ccct***ag***ATATGT	GAGCAG***gt***agca	53	1886
54	113	271236-271348	attc***ag***GGGATC	GATTGG***gt***atgt	54	>14472
55	155	285921-286075	atgc***ag***GCACCC	ATTAAC***gt***aagt	55	629
56	171	286704-286874	tgta***ag***GACATC	GTGAAG***gt***agga	56	5979
57	137	292854-292990	ttgc***ag***GCCTAC	CCGCAG***gt***gaga	57	1755
58	663	294746-295408[b]	cctc***ag***CTTGGT			

[a]Start codon: 80874-80876, [b]stop codon: 294867-294869.

Table 2 Exon/intron organization of the porcine *ACACB* gene.

Exon	Size (bp)	Acc. No. EU853705	3' splice acceptor	5' splice donor	Intron	Size (bp)
1a	115	9246-9360		GGCCAG***gt***aagt	1a	10816
1b	61	20175-20236		GTGACC***gt***aagt	1b	5334
2	656	25571-26226[a]	ttac***ag***ATTTTC	GATAAG***gt***aaca	2	9475
2a	139	35702-35840		AGTAAA***gt***aagt	2a	11412
3	127	47253-47379	tggc***ag***GCCCAG	GAGAAG***gt***acag	3	2358
4	139	49738-49876	gtcc***ag***GTGCTC	ATGCAG***gt***actt	4	1692
5	110	51569-51678	ttcc***ag***AGTATA	GTGCAG***gt***aagt	5	209
6	82	51888-51969	ttgc***ag***GCAGTG	TCTTAG***gt***agag	6	1147
7	99	53117-53215	ctcc***ag***GTCCTC	GAAGTG***gt***aaga	7	1445
8	110	54661-54770	cccc***ag***GTCTGA	TTGGAG***gt***aaac	8	106
9	111	54877-54987	ccac***ag***GCAGCA	AGACAG***gt***gagc	9	2545
10	210	57535-57744	cccc***ag***GTGCAG	GAGCAG***gt***gggt	10	272
11	171	58017-58187	ctgc***ag***TGTGCT	CTACAG***gc***aagg	11	4306
12	162	62494-62655	tttc***ag***ATCGCC	GATGAG***gc***aagtt	12	1801
13	164	64457-64620	ctcc***ag***GGGTTT	TATTTC***gt***cagt	13	919
14	151	65540-65690	ctca***ag***GAACAT	GTGCAG***gt***gggg	14	90
15	104	65781-65884	tcct***ag***GCCGAG	GGAAAG***gt***ggga	15	1558
16	82	67443-67524	tgtt***ag***GGGCCA	CTTAAG***gt***aagg	16	4145
17	146	71670-71815	ccac***ag***GTGGCC	CAACAG***gt***gtgt	17	1141
18	151	72957-73107	ctcc***ag***TTACCG	ATAGAG***gt***aggc	18	1244
19	135	74352-74486	aaac***ag***GTGATG	CACCCA***gt***atgt	19	1799
20	147	76286-76432	tgtc***ag***GCTGAG	ATGAAG***gt***gggt	20	662
21	189	77095-77283	ctgc***ag***CTGAAG	CAGCAG***gt***atgt	21	2522
22	101	79806-79906	atcc***ag***ATAGCC	CCAAAA***gt***gagt	22	787
23	89	80694-80782	cccc***ag***ATACCG	AACAAG***gc***aaga	23	92
24	125	80875-80999	ttcc***ag***CCCATT	TTGATC***gt***aagc	24	4520
25	114	85520-85633	ccaa***ag***GATGAG	AGGCAG***gt***aggg	25	143
26	114	85777-85890	ctgc***ag***GTCCTG	CTCAAG***gt***gagg	26	596
27	90	86487-86576	ctgt***ag***AAACTA	TTGGAG***gt***aagt	27	3231
28	119	89808-89926	catt***ag***GTTTAC	AAACCG***gt***acag	28	1712
29	138	91639-91776	ctgc***ag***GATGGC	CATCAG***gt***accc	29	498
30	100	92275-92374	ccac***ag***GAACTT	AGCAAG***gt***gagt	30	84
31	108	92459-92566	tccc***ag***AGCCTC	TCCAAG***gt***acgg	31	979
32	57	93546-93602	atgc***ag***AAAAAC	CAAGAG***gt***cagt	32	259
33	42	93862-93903	ttgc***ag***AAAGAA	GACAAG***gt***atgg	33	1997
34	216	95901-96116	ccgc***ag***TTTGCA	ACCAAG***gt***agga	34	1177
35	156	97294-97449	tggc***ag***GAGGCC	ACCAAG***gt***atcc	35	854
36	204	98304-98507	acac***ag***ATCGAG	GGAAAC***gt***aagg	36	847
37	156	99355-99510	gtcc***ag***ATCATG	AGGCAG***gc***aagt	37	5246
38	123	104757-104879	ctgt***ag***GCTCTT	AACGAG***gg***aaga	38	1131
39	270	106011-106280	ctcc***ag***GTGGGC	TACAAG***gt***acgt	39	1554
40	98	107835-107932	tttt***ag***GGATTT	ATCCAG***gt***aaat	40	1679
41	121	109612-109732	gtga***ag***GTACAT	AGCCTG***gt***gagt	41	446
42	111	110179-110289	ccac***ag***GTGACC	AACAAG***gt***gacc	42	1244
43	144	111534-111677	aagc***ag***GTCCTG	CCAAAG***gt***gctg	43	937
44	121	112615-112735	ttcc***ag***GACAAT	ACCCAA***gt***aagt	44	1868
45	97	114604-114700	acac***ag***CTCTAA	AGCCAG***gt***aacc	45	2361
46	97	117062-117158	ttgc***ag***GCTAGG	GCCAAG***gt***tagg	46	1124
47	136	118283-118418	ttcc***ag***ATAATC	TGAAAG***gt***aagc	47	1266
48	178	119685-119862	tttc***ag***ACATGT	GAGCAG***gt***gggt	48	3464
49	113	123327-123439	tttc***ag***GGCAAG	AGCTAG***gt***gagt	49	1785
50	155	125225-125379	cctc***ag***GATCGT	ATCTGT***gt***aaga	50	684
51	171	126064-126234	ctgc***ag***GATATC	GTCAAG***gt***gggc	51	113
52	137	126348-126484	cccc***ag***GCCTAC	CCGAGG***gt***gagt	52	649
53	1483	127134-128616[b]	ctgc***ag***CCTGGT			

[a]Start codon: 25580-25582, [b]stop codon: 127258-127260.

Table 3 Exon/intron organization of the porcine *CPT1A* gene.

Exon	Size (bp)	Acc. No. FJ263681	3' splice acceptor	5' splice donor	Intron	Size (bp)
1	163	62175-62337		GACTCG***gt***aggg	1	15814
2	154	78152-78305[a]	ctcc***ag***GCTACT	TTCAAG***gt***gagg	2	1086
3	140	79392-79531	cggt***ag***AATGG	CGCCAC***gt***aagg	3	6449
4	172	85981-86152	cggc***ag***CGGCTA	TGGATG***gt***gagg	4	2226
5	102	88379-88480	taac***ag***ATGATG	AGCAGG***gt***aggt	5	2506
6	138	90987-91124	tccc***ag***TACCTG	AATTAC***gt***gagt	6	764
7	78	91889-91966	tgac***ag***GTGAGC	GCCATG***gt***gagt	7	1943
8	108	93910-94017	tgtc***ag***GATCTG	AAACCG***gt***aagc	8	911
9	85	94929-95013	ttgc***ag***ATTCTC	AAACAG***gt***gcgc	9	926
10	196	95938-96133	ccac***ag***ACACCA	GGACAG***gt***aggc	10	2509
11	189	98643-98831	ctgc***ag***GGTGCC	TGACAG***gt***atca	11	1167
12	106	99999-100104	ccgt***ag***GTGGTT	TGGGAG***gt***gagt	12	4137
13	117	104242-104358	tgac***ag***TATGTC	GAGGAG***gt***aggt	13	2441
14	165	106800-106964	cgcc***ag***TGTCAG	TACAAG***gt***gaga	14	7097
15	135	114062-114196	cccc***ag***GACATG	CAGCCG***gt***acgt	15	1910
16	153	116107-116259	tctt***ag***GTGGAA	AAGGAG***gt***gtgt	16	1141
17	114	117401-117514	taat***ag***GTCCTG	GGACCG***gt***aagt	17	713
18	93	118228-118320	ccgt***ag***GTTGCT	GAGACG***gt***acgt	18	1042
19	2056	119363-121418[b]	tgaa***ag***GATTCT			

[a]Start codon: 78165-78167, [b]stop codon: 119447-119449.

Table 4 Exon/intron organization of the porcine *CPT2* gene.

Exon	Size (bp)	Acc. No. FJ263683	3' splice acceptor	5' splice donor	Intron	Size (bp)
1	616	20682-21297[a]		GCCCAG***gt***gagc	1	12386
2	81	33684-33764	tccc***ag***GCTGCC	GTTCAG***gt***aaat	2	2346
3	107	36111-36217	tttc***ag***GAAAAC	TTTCAG***gt***gggt	3	3967
4	1305	40185-41489	tttt***ag***GCCCCT	CCATGG***gt***aagc	4	1520
5	452	43010-43461[b]	tgac***ag***GCCAGG			

[a]Start codon: 21146-21148, [b]stop codon: 43339-43341.

Table 5 Exon/intron organization of the porcine *MLYCD* gene.

Exon	Size (bp)	Acc. No. FJ263687	3' splice acceptor	5' splice donor	Intron	Size (bp)
1	585	1731-2315[a]		GTCCGG***gt***aagg	1	8090
2	113	10406-10518	ttgc***ag***GAAATG	TAGCAA***gt***aagt	2	1008
3	157	11527-11683	cgtc***ag***GTCTGA	ATCCAG***gt***acct	3	2027
4	153	13711-13863	ttcc***ag***ACGATC	CTGCAG***gt***gggt	4	1936
5	839	15800-16638[b]	cagc***ag***AAGGAG			

[a]Start codon: 1773-1775, [b]stop codon: 16331-16333.

Table 6 Exon/intron organization of the porcine *CHKB* and *CPT1B* genes.

CHKB						
Exon	Size (bp)	Acc. No. FJ263682	3' splice acceptor	5' splice donor	Intron	Size (bp)
1	536	8246-8781[a]		CGTGAG***gt***agga	1	215
2	109	8997-9105	ctgc***ag***CGGAGG	CTGCAG***gt***gagg	2	278
3	114	9384-9497	tttc***ag***GGCGTG	ATCCCA***gt***atga	3	180
4	134	9678-9811	gtgc***ag***AGCCGG	GGAGCG***gt***gagt	4	267
5	96	10079-10174	cttt***ag***GTATTT	CCTCAG***gt***gagg	5	181
6	59	10356-10414	ctcc***ag***GAAGTT	AGGAAG***gt***agga	6	87
7	82	10502-10583	ctcc***ag***GGAACA	CTACAG***gt***gagg	7	108
8	109	10692-10800	cccc***ag***GGGCTT	CAGCAG***gt***atgt	8	142
9	104	10943-11046	tttc***ag***CTCCAT	TAATCG***gt***gagg	9	243
10	82	11290-11371	cctc***ag***GTACGC	TACTTG***gt***aagt	10	135
11	278	11507-11784[b]	tcct***ag***GAGTAT			
CPT1B						
Exon	Size (bp)	Acc. No. FJ263682	3' splice acceptor	5' splice donor	Intron	Size (bp)
1	109	12314-12422		CCTTCT***gt***gagt	1	345
2	160	12768-12927[c]	tccc***ag***GTGCTG	ATCAAG***gt***gggt	2	340
3	140	13268-13407	ccac***ag***AATGGC	TGAGAG***gt***aaaa	3	300
4	178	13708-13885	tgac***ag***ATATGG	TGGGCT***gt***gagc	4	267
5	102	14153-14254	cccc***ag***ATCTGT	CATCGG***gt***gagg	5	177
6	138	14432-14569	ggat***ag***TACCTA	AACTAT***gt***gagt	6	78
7	78	14648-14725	ccgc***ag***GTGAGT	GTCATG***gt***aaga	7	818
8	108	15544-15651	tggc***ag***GACCTG	AAGCCT***gt***gagt	8	74
9	85	15726-15810	tccc***ag***GTGATG	ACACAG***gt***actg	9	547
10	196	16358-16553	tccc***ag***ACACAC	GGGAAG***gt***agtg	10	336
11	186	16890-17075	cccc***ag***AGTGGA	CAACAG***gt***accc	11	85
12	106	17161-17266	ctgc***ag***GTGGTT	TGGGAG***gt***aaga	12	87
13	117	17354-17470	ccgc***ag***TTCGTC	GAGCAG***gt***gtgt	13	401
14	165	17872-18036	cggc***ag***TGCCAG	TTCCGG***gt***agga	14	72
15	135	18109-18243	cccc***ag***GACAGG	CGCGTG***gt***gagc	15	95
16	153	18339-18491	cccc***ag***AAAGCA	GCTGAG***gt***cagt	16	537
17	114	19029-19142	ttgc***ag***GTGCTC	GGCCCT***gt***aagt	17	469
18	93	19612-19704	cccc***ag***GTGGCC	GAGACG***gt***gagt	18	112
19	335	19817-20151[d]	gcgc***ag***AACGCC			

[a]*CHKB* - choline kinase beta enzyme gene, start codon: 8558-8560, [b]stop codon: 11579-11581, [c]start codon: 12787-12789, [d]stop codon: 19898-19900.

Table 7 Exon/intron organization of the porcine *FADS1* gene.

Exon	Size (bp)	Acc. No. FJ263684	3'splice acceptor	5' splice donor	Intron	Size (bp)
1	192	7018-7209[a]		GCTACG***gt***gagc	1	2134
2	111	9344-9454	ttgc***ag***GATCCC	AAGAAT***gt***gagt	2	580
3	198	10035-10232	ctac***ag***AAAGAG	GTTCAG***gt***gaga	3	830
4	102	11063-11164	gccc***ag***GCCCAG	CTGAAG***gt***cagt	4	99
5	129	11264-11392	ctgt***ag***GGGCCC	GTGGAG***gt***gagc	5	3774
6	61	15167-15227	tcac***ag***CTTGGG	TCTTGA***gt***gagt	6	>1675
7	77	17003-17079	cccc***ag***TTGGGC	TGGGTG***gt***aagt	7	700
8	98	17780-17877	ccgc***ag***GACTTG	GGTCAG***gt***agga	8	215
9	97	18093-18189	cacc***ag***GTTCCT	ATGCAG***gt***gagg	9	224
10	80	18414-18493	ctcc***ag***GTCCGG	GCACCA***gt***gagt	10	110
11	126	18604-18729	ctgc***ag***TCTTTT	TGTCCA***gt***gagt	11	333
12	2318	19063-21380[b]	ccac***ag***CTCACT			

[a]Start codon: 7018-7020, 5'UTR could not be determined, [b]stop codon: 19112-19114.

Table 8 Exon/intron organization of the porcine *FADS2* gene.

Exon	Size (bp)	Acc. No. FJ263685	3' splice acceptor	5' splice donor	Intron	Size (bp)
1	242	8139-8380[a]		GCTACG***gt***aagg	1	9990
2	111	18371-18481	cccc***ag***GATGCC	AAGAAC***gt***gagt	2	>1784
3	198	20366-20563	caac***ag***TCTGAG	TCTCAG***gt***gagg	3	116
4	102	20680-20781	cccc***ag***GCCCAG	TTAAAG***gt***aaat	4	>7451
5	126	28333-28458	ccac***ag***GGTGCC	GTTGAG***gt***agga	5	>5205
6	61	33764-33824	tctc***ag***TACGGC	TCCTGA***gt***gagt	6	365
7	77	34190-34266	ctgc***ag***TTGGGC	TGGGTG***gt***gagt	7	5764
8	98	40031-40128	ccgc***ag***GACTTG	TATCAG***gt***gcct	8	221
9	97	40350-40446	ttcc***ag***GTTCCT	ACCCAG***gt***gagg	9	336
10	80	40783-40862	cgac***ag***CTGGCA	GCACCA***gt***gagt	10	447
11	126	41310-41435	ccct***ag***CCTCTT	CATCGG***gt***aagg	11	282
12	171	41718-41888[b]	tccc***ag***GTCCCT			

[a]Start codon: 8174-8176, [b]stop codon: 41767-41769.

Table 9 Exon/intron organization of the porcine *FADS3* gene.

Exon	Size (bp)	Acc. No. FJ263686	3' splice acceptor	5' splice donor	Intron	Size (bp)
1	386	2765-3150[a]		GCCACG***gt***aagg	1	12904
2	111	16055-16165	cttc***ag***GATGCC	CAGAAT***gt***gagc	2	653
3	198	16819-17016	tgcc***ag***GCCCAG	TCCCAG***gt***gacc	3	279
4	102	17296-17397	cccc***ag***GCCCAG	CTGAAA***gt***gagg	4	111
5	123	17509-17631	ctgc***ag***GGTTTC	ATCGAG***gt***gcat	5	255
6	61	17887-17947	ccac***ag***TACGGC	TCCTGA***gt***gagt	6	1088
7	77	19036-19112	ttgc***ag***TCGGCC	TGGACG***gt***gagt	7	460
8	98	19573-19670	ctgc***ag***GACCTG	TGTCAG***gt***atgg	8	344
9	97	20015-20111	tccc***ag***GGTCCT	TCCCAG***gt***gggc	9	121
10	80	20233-20312	tggc***ag***TTGGCA	GCACCA***gt***gagc	10	168
11	126	20481-20606	tccc***ag***CCTCTT	CATTAG***gt***aagc	11	1533
12	394	22140-22533[b]	accc***ag***GTCCCT			

[a]Start codon: 2944-2946, [b]stop codon: 22189-22191.

Table 10 Exon/intron organization of the porcine *SCD5* gene.

Exon	Size (bp)	Acc. No. FJ263688	3' splice acceptor	5' splice donor	Intron	Size (bp)
1	433	26794-27226[a]		TCTGGG***gt***aagt	1	67355
2	131	94582-94712	ttgc***ag***CCTACT	TTCCAG***gt***gggg	2	19999
3	206	114712-114917	ctgc***ag***AACGAC	GAGAAA***gt***aagt	3	33076
4	233	147994-148226	ttcc***ag***GTACTA	CCATCG***gt***gagt	4	4003
5	1470	152230-153699[b]	ttcc***ag***GTGAAGG			

[a]Start codon: 26989-26991, [b]stop codon: 152418-152420.

Table 11 Exon/intron organization of the porcine *PRKAA2* gene.

Exon	Size (bp)	Acc. No. EU853704	3' splice acceptor	5' splice donor	Intron	Size (bp)
1	144	16333-16476[a]		TGAAGA***gt***tgag	1	>29508
2	142	45584-45725	ctct***ag***TTGGAG	CAAACT***gt***aagt	2	>6988
3	94	52313-52406	aaaa***ag***ATACCA	GGACGG***gt***gagt	3	1072
4	145	53478-53622	tgat***ag***GTTGAA	ATTTTG***gt***atgt	4	2009
5	88	55631-55718	tttc***ag***GATTAT	AGGCAG***gt***aaaa	5	2238
6	225	57956-58180	ctct***ag***ATTGTA	CATAAG***gt***gaat	6	>15181
7	505	72961-73465	tcct***ag***AGAGCA	TGGAAG***gt***agga	7	1348
8	127	74813-74939	tttt***ag***GTAGTG	TTGATG***gt***aagg	8	1732
9	676	76671-77346[b]	tttc***ag***ATGAGG			

[a]Start codon: 16383-16385, [b]stop codon: 76907-76909.

Table 12 Sequence variants identified in the porcine *ACACA* gene.

Gene symbol	No.	Variant location	SNP_id	Region	Allele 1	Allele 2	Effect
ACACA	AA1	76934-59		Intron 3	$(CT)_n$	$(CT)_m$	
(FJ263680)	AA2	127389	1751	Intron 13	C	G	
	AA3	131112	1748	Intron 13	C	T	
	AA4	131434	1749	Exon 14	C	G	Synonymous (Leu)
	AA5	131450	1750	Intron 14	C	G	
	AA6	132693	1449	Intron 15	C	T	
	AA7	132737	1450	Intron 15	A	G	
	AA8	132976	1728	Intron 15	A	G	
	AA9	132995	1729	Intron 15	C	T	
	AA10	133025	1730	Intron 15	G	T	
	AA11	133219	1731	Exon 16	C	T	Synonymous (Ser)
	AA12	133397	1732	Intron 16	A	C	
	AA13	137631	1733	Exon 18	C	G	Synonymous (Val)
	AA14	137842	1734	Intron 18	C	T	
	AA15	142703	1736	Exon 21	C	T	Synonymous (Ile)
	AA16	142802	1737	Exon 21	G	T	Synonymous (Gly)
	AA17	144069	1738	Intron 22	A	T	
	AA18	144171	1739	Intron 22	C	T	
	AA19	144260	1740	Intron 22	A	G	
	AA20	144361	1741	Intron 22	G	T	
	AA21	144579	1742	Intron 23	A	G	
	AA22	144664	1743	Intron 23	A	G	
	AA23	153738	1856	Intron 27	A	C	
	AA24	153742	1857	Intron 27	C	T	
	AA25	178720	1451	Intron 32	A	G	
	AA26	199072	1679	Intron 40	C	T	
	AA27	223060	2242	Intron 42	C	T	
	AA28	223372	2243	Exon 42	C	T	Synonymous (His)
	AA29	227756_7ins	1524	Intron 44	Ins (280bp)	Del	
	AA30	228313	1462	Intron 44	C	T	
	AA31	228477	1463	Intron 45	C	T	
	AA32	228587	1464	Intron 45	A	G	
	AA33	228613	1465	Intron 45	C	G	
	AA34	251928	1804	Intron 47	A	G	
	AA35	251998	1805	Exon 48	C	G	Synonymous (Gly)
	AA36	252028	1806	Exon 48	C	T	Synonymous (Leu)
	AA37	253494	1807	Exon 49	A	G	Synonymous (Pro)
	AA38	256873	2229	Exon 50	A	G	Synonymous (Gln)
	AA39	256939	2230	Exon 50	A	G	Synonymous (Val)
	AA40	260345	1808	Exon 51	C	T	Synonymous (Ala)
	AA41	261352	1809	Exon 52	A	G	Synonymous (Ala)
	AA42	261638	1810	Intron 52	C	T	
	AA43	261650	1811	Intron 52	C	G	
	AA44	269233	1812	Exon 53	A	G	Synonymous (Ser)
	AA45	269392	1813	Intron 53	C	T	
	AA46	269460	1815	Intron 53	G	T	
	AA47	269464	1816	Intron 53	A	G	
	AA48	269478	1817	Intron 53	A	G	
	AA49	269483	1818	Intron 53	C	T	
	AA50	271263	2293	Exon 54	C	T	Synonymous (Thr)
	AA51	271338	2244	Exon 54	C	T	Synonymous (Ala)
	AA52	285656	1821	Intron 54	A	G	
	AA53	292670	1819	Intron 56	G	T	
	AA54	292770	1820	Intron 56	C	T	
	AA55	294888	1830	3’ UTR	A	G	
	AA56	294917	1831	3’ UTR	A	G	
	AA57	294928	1832	3’ UTR	C	T	
	AA58	294968	1833	3’ UTR	A	T	
	AA59	295053	1834	3’ UTR	C	T	
	AA60	295086	1835	3’ UTR	C	T	
	AA61	295127	1836	3’ UTR	C	T	
	AA62	295210	1837	3’ UTR	A	G	

Table 13 Sequence variants identified in the porcine *ACACB* gene.

Gene symbol	No.	Variant location	SNP_id	Region	Allele 1	Allele 2	Effect
ACACB	AB1	8142	1700	5’ end	A	T	
(EU853705)	AB2	8224	1701	5’ end	A	G	
	AB3	8343	1702	5’ end	A	G	
	AB4	8345	1703	5’ end	C	T	
	AB5	9007	1584	5’ end	A	G	
	AB6	9144	1567	Exon 1a	C	T	
	AB7	9396	1568	Intron 1a	C	T	
	AB8	9432	1569	Intron 1a	A	C	
	AB9	20124	1570	Promoter II	A	G	
	AB10	20248	1571	Intron 1b	C	T	
	AB11	20321	1572	Intron 1b	G	T	
	AB12	20326	1573	Intron 1b	T	C	
	AB13	20353	1574	Intron 1b	A	T	
	AB14	20362	1575	Intron 1b	A	G	
	AB15	25835	1576	Exon 2	A	G	Ser86Gly
	AB16	25926	1577	Exon 2	A	G	Ser116Asn
	AB17	25963	1578	Exon 2	A	C	Synonymous (Ala)
	AB18	26000	1579	Exon 2	A	C	Synonymous (Arg)
	AB19	26032	1580	Exon 2	A	G	Synonymous (Lys)
	AB20	26053	1581	Exon 2	A	G	Synonymous (Gln)
	AB21	47415	1582	Intron 3	C	T	
	AB22	47461	1583	Intron 3	C	T	
	AB23	51391	1628	Intron 4	C	T	
	AB24	51432	1629	Intron 4	C	T	
	AB25	51448	1630	Intron 4	C	T	
	AB26	51484	1631	Intron 4	C	T	
	AB27	51718	1632	Intron 5	C	T	
	AB28	51859	1633	Intron 5	C	T	
	AB29	51927	1634	Intron 6	A	G	
	AB30	51937	1635	Intron 6	C	G	
	AB31	53074	1613	Intron 6	A	C	
	AB32	53095	1614	Intron 6	G	T	
	AB33	54830	1627	Intron 8	A	G	
	AB34	57633	1636	Exon 10	C	T	Synonymous (Asp)
	AB35	57672	1637	Exon 10	A	G	Synonymous (Arg)
	AB36	57754	1638	Intron 10	A	G	
	AB37	57773	1639	Intron 10	A	G	
	AB38	57812	1640	Intron 10	C	T	
	AB39	57956	1641	Intron 10	C	T	
	AB40	57959	1642	Intron 10	A	C	
	AB41	57965	1643	Intron 10	C	T	
	AB42	58073	1644	Exon 11	C	T	Synonymous (Tyr)
	AB43	62398	1615	Intron 11	C	T	
	AB44	62428	1616	Intron 11	C	T	
	AB45	62429	1617	Intron 11	A	G	
	AB46	62440	1618	Intron 11	C	T	
	AB47	64370	1619	Intron 12	C	T	
	AB48	64374	1620	Intron 12	A	G	
	AB49	64408	1621	Intron 12	A	G	
	AB50	64409	1622	Intron 12	C	G	
	AB51	65678	1623	Exon 14	C	T	Synonymous (Ala)
	AB52	65717	1624	Intron 14	A	G	
	AB53	65747	1625	Intron 14	C	T	
	AB54	65786	1626	Exon 15	A	G	Synonymous (Glu)
	AB55	67382	1711	Intron 15	C	T	
	AB56	67440	1712	Intron 15	C	T	
	AB57	67500	1713	Exon 16	C	T	Synonymous (Tyr)
	AB58	71636	1659	Intron 16	A	G	
	AB59	71660	1660	Intron 16	A	G	
	AB60	71825	1661	Intron 17	G	T	

Table 13 Sequence variants identified in the porcine *ACACB* gene (continued).

Gene symbol	No.	Variant location	SNP_id	Region	Allele 1	Allele 2	Effect
ACACB	AB61	71829	1662	Intron 17	G	T	
(EU853705)	AB62	71948	1663	Intron 17	G	T	
	AB63	73056	1664	Exon 18	A	G	Synonymous (Thr)
	AB64	73158	1665	Intron 18	A	G	
	AB65	74151	1666	Intron 19	A	G	
	AB66	74158	1667	Intron 19	A	G	
	AB67	80703	1704	Exon 23	C	T	Synonymous (Ser)
	AB68	80712	1705	Exon 23	C	T	Synonymous (Arg)
	AB69	80921	1706	Exon 24	A	G	Synonymous (Pro)
	AB70	80999	1707	Exon 24	C	T	Synonymous (Ile)
	AB71	85646	1708	Intron 25	A	G	
	AB72	85732	1709	Intron 25	C	T	
	AB73	85748	1710	Intron 25	C	T	
	AB74	91580	1684	Intron 28	A	G	
	AB75	91605	1685	Intron 28	G	T	
	AB76	95964	1689	Exon 34	A	C	Synonymous (Arg)
	AB77	96126	1690	Intron 34	C	T	
	AB78	96160	1691	Intron 34	C	T	
	AB79	96189	1692	Intron 34	C	T	
	AB80	96224	1693	Intron 34	A	G	
	AB81	99674	1714	Intron 37	C	T	
	AB82	99798	1715	Intron 38	A	G	
	AB83	106106	1675	Exon 39	C	T	Synonymous (Ile)
	AB84	106214	1676	Exon 39	C	T	Synonymous (Ile)
	AB85	107808	1674	Intron 39	C	T	
	AB86	109757	1677	Intron 41	A	C	
	AB87	109765	1678	Intron 41	A	C	
	AB88	112446	1688	Intron 43	C	T	
	AB89	114727	1716	Intron 45	A	G	
	AB90	117026	1717	Intron 45	C	T	
	AB91	118453	1718	Intron 47	A	G	
	AB92	119633	1744	Intron 47	A	G	
	AB93	119692	1745	Exon 48	C	T	Synonymous (Tyr)
	AB94	119821	1746	Exon 48	C	T	Synonymous (Ile)
	AB95	125250	1668	Exon 50	C	T	Synonymous (Asp)
	AB96	125319	1669	Exon 50	A	G	Synonymous (Ala)
	AB97	125986	1719	Intron 50	G	T	
	AB98	126084	1720	Exon 51	C	T	Synonymous (Thr)
	AB99	126262	1721	Intron 51	A	G	
	AB100	126303	1722	Intron 51	A	G	
	AB101	127056	1680	Intron 52	A	G	
	AB102	127321	1681	3’ UTR	A	G	
	AB103	127330	1682	3’ UTR	C	T	
	AB104	127342	1683	3’ UTR	C	T	

Table 14 Sequence variants identified in the porcine *CPT1* genes.

Gene symbol	No.	Variant location	SNP_id	Region	Allele 1	Allele 2	Effect
CPT1A	CA1	86183	1757	Intron 4	C	G	
(FJ263681)	CA2	86220	1758	Intron 4	C	T	
	CA3	88471	2256	Exon 5	A	G	Synonymous (Thr)
	CA4	88502	2182	Intron 5	C	T	
	CA5	91257-8		Intron 6	Ins (G)	Del	
	CA6	91774	1764	Intron 6	C	G	
	CA7	91815	1765	Intron 6	A	G	
	CA8	91842	1766	Intron 6	C	T	
	CA9	92016	1767	Intron 7	A	G	
	CA10	92036	1768	Intron 7	C	G	
	CA11	93841	2257	Intron 7	C	G	
	CA12	93988	2258	Exon 8	A	C	Synonymous (Arg)
	CA13	95093	2259	Intron 9	A	C	
	CA14	95095	2260	Intron 9	A	G	
	CA15	95132	2261	Intron 9	C	T	
	CA16	95139	2262	Intron 9	C	T	
	CA17	95197	2263	Intron 9	C	T	
	CA18	95248	2264	Intron 9	A	C	
	CA19	95264	2265	Intron 9	A	G	
	CA20	95278	2266	Intron 9	C	T	
	CA21	95279	2267	Intron 9	G	T	
	CA22	95315	2268	Intron 9	C	T	
	CA23	95847	1752	Intron 9	A	G	
	CA24	95869	1753	Intron 9	A	G	
	CA25	95956	1754	Exon 10	A	G	Gln328Arg
	CA26	95960	1755	Exon 10	C	T	Synonymous (Asp)
	CA27	96150	1756	Intron 10	C	T	
	CA28	100026	1759	Exon 12	C	T	Synonymous (Ile)
	CA29	100215	1760	Intron 12	C	T	
	CA30	104374	1761	Intron 13	C	T	
	CA31	104403	1762	Intron 13	C	T	
	CA32	104406	1763	Intron 13	C	T	
	CA33	106837	2172	Exon 14	A	G	Asn537Ser
	CA34	106970	2173	Intron 14	A	T	
	CA35	106982	2174	Intron 14	C	T	
	CA36	107010	2175	Intron 14	A	C	
	CA37	107042	2176	Intron 14	C	T	
	CA38	117386	1747	Intron 16	G	T	
CPT1B	CB1	12242	2240	5’ end	A	C	
(FJ263682)	CB2	12527	2241	Intron 1	A	G	
	CB3	19124	2238	Exon 17	C	T	Synonymous (Ala)
	CB4	19188	2239	Inton 17	A	T	

Table 15 Sequence variants identified in the porcine *CPT2* gene.

Gene symbol	No.	Variant location	SNP_id	Region	Allele 1	Allele 2	Effect
CPT2	CC1	19257	1871	5' end	A	G	
(FJ263683)	CC2	19285	1872	5' end	C	T	
	CC3	19296	1873	5' end	C	T	
	CC4	19324	1874	5' end	G	T	
	CC5	19381	1875	5' end	C	G	
	CC6	19791	1849	5' end	A	G	
	CC7	19939	1850	5' end	C	G	
	CC8	20495	1539	5' end	A	T	
	CC9	20876	1547	Exon 1	C	G	
	CC10	20941	1548	Exon 1	A	G	
	CC11	21149	1543	Exon 1	A	G	Met2Val
	CC12	21183	1544	Exon 1	A	G	Asp13Gly
	CC13	21328	1545	Intron 1	A	G	
	CC14	33711	1540	Exon 2	C	T	Synonymous (Thr)
	CC15	33784	1541	Intron 2	C	G	
	CC16	33818	1542	Intron 2	C	T	
	CC17	36038	1537	Intron 2	A	G	
	CC18	36077	1538	Intron 2	A	G	
	CC19	40451	1452	Exon 4	A	G	Gly203Ser
	CC20	40642	1453	Exon 4	C	T	Synonymous (Thr)
	CC21	40942	1454	Exon 4	A	G	Synonymous (Thr)
	CC22	41033	1549	Exon 4	A	G	Ile397Val
	CC23	41107	1550	Exon 4	C	T	Synonymous (Asp)
	CC24	41431	1551	Exon 4	A	G	Synonymous (Arg)
	CC25	43050	1546	Exon 5	A	G	Synonymous (Leu)

Table 16 Sequence variants identified in the porcine *MLYCD* gene.

Gene symbol	No.	Variant location	SNP_id	Region	Allele 1	Allele 2	Effect
MLYCD	MD1	837-864		5' end	$(CA)_n(CG)_m$		
(FJ263687)	MD2	1278	1510	5' end	A	C	
	MD3	1298		5' end	Ins (A)	Del	
	MD4	1349	1511	5' end	A	G	
	MD5	1366	1512	5' end	C	G	
	MD6	1378	1513	5' end	C	T	
	MD7	1431	1514	5' end	A	G	
	MD8	1436	1515	5' end	C	T	
	MD9	1456	1516	5' end	A	G	
	MD10	1496	1851	5' end	G	T	
	MD11	1598	1466	5' end	C	T	
	MD12	1619	1467	5' end	A	G	
	MD13	1638	1468	5' end	A	C	
	MD14	10179	1415	Intron 1	C	T	
	MD15	10237	1416	Intron 1	A	T	
	MD16	10314	1417	Intron 1	A	G	
	MD17	10315	1418	Intron 1	C	T	
	MD18	10456	1419	Exon 2	C	G	Synonymous (Gly)
	MD19	10492	1420	Exon 2	A	G	Synonymous (Pro)
	MD20	10513	1421	Exon 2	C	T	Synonymous (Ile)
	MD21	10529	1585	Intron 2	C	T	
	MD22	10536	1586	Intron 2	A	C	
	MD23	10562	1587	Intron 2	C	T	
	MD24	10563	1588	Intron 2	A	G	
	MD25	10606	1589	Intron 2	G	T	
	MD26	10625	1590	Intron 2	A	G	
	MD27	10747	1591	Intron 2	C	T	
	MD28	10759	1592	Intron 2	A	G	
	MD29	10792	1593	Intron 2	A	C	
	MD30	10799	1594	Intron 2	A	G	
	MD31	10813	1595	Intron 2	A	G	
	MD32	10825	1596	Intron 2	A	G	
	MD33	10866	1597	Intron 2	C	T	
	MD34	10888	1598	Intron 2	A	G	
	MD35	13601	1469	Intron 3	A	G	
	MD36	13633	1470	Intron 3	C	G	
	MD37	13652	1471	Intron 3	A	G	
	MD38	13713	1472	Exon 4	A	G	Synonymous (Thr)
	MD39	13737	1473	Exon 4	A	G	Synonymous (Pro)
	MD40	13770	1599	Exon 4	A	G	Synonymous (Thr)
	MD41	13875	1474	Intron 4	A	G	
	MD42	13897	1475	Intron 4	C	G	
	MD43	13900	1476	Intron 4	A	G	
	MD44	13937	1601	Intron 4	A	G	
	MD45	13959	1477	Intron 4	A	C	
	MD46	13962	1600	Intron 4	C	G	
	MD47	13994	1478	Intron 4	A	G	
	MD48	13998	1479	Intron 4	A	G	
	MD49	14033	1480	Intron 4	A	G	
	MD50	14041	1481	Intron 4	A	G	
	MD51	15746	1482	Intron 4	A	G	

Table 16 Sequence variants identified in the porcine *MLYCD* gene (continued).

Gene symbol	No.	Variant location	SNP_id	Region	Allele 1	Allele 2	Effect
MLYCD	MD52	15785	1483	Intron 4	A	G	
(FJ263687)	MD53	15808	1484	Exon 5	C	T	Synonymous (Phe)
	MD54	15925	1367	Exon 5	C	T	Synonymous (Ala)
	MD55	15996	1368	Exon 5	A	G	Asp388Gly
	MD56	16093	1485	Exon 5	C	T	Synonymous (Tyr)
	MD57	16162	1486	Exon 5	C	T	Synonymous (Gly)
	MD58	16216	1487	Exon 5	A	C	Synonymous (Arg)
	MD59	16238	1488	Exon 5	A	G	Ala469Thr
	MD60	16264	1489	Exon 5	C	G	Synonymous (Ser)
	MD61	16282	1490	Exon 5	A	C	Synonymous (Ser)
	MD62	16357	1491	3’ UTR	A	G	
	MD63	16367_8ins		3’ UTR	Ins (C)	Del	
	MD64	16372	1492	3’ UTR	C	T	
	MD65	16707	1517	3’ UTR	C	T	
	MD66	16769	1518	3’ UTR	A	G	
	MD67	16786	1519	3’ UTR	A	G	
	MD68	16789	1520	3’ UTR	G	T	

Table 17 Sequence variants identified in the porcine desaturase genes.

Gene symbol	No.	Variant location	SNP_id	Region	Allele 1	Allele 2	Effect
FADS1	FS1	6702	1371	5' end	A	G	
(FJ263684)	FS2	9533	1141	Intron 2	C	T	
	FS3	9670	1142	Intron 2	A	G	
	FS4	10262	1143	Intron 3	A	G	
	FS5	11224	1235	Intron 4	A	G	
	FS6	11261	1236	Intron 4	C	T	
	FS7	11305	1237	Exon 5	C	T	Synonymous (His)
	FS8	15121	1234	Intron 5	A	G	
	FS9	17945	1034	Intron 8	C	T	
	FS10	17958	1035	Intron 8	A	G	
	FS11	18008	1036	Intron 8	A	G	
	FS12	18030	1037	Intron 8	C	G	
	FS13	18045	1038	Intron 8	C	T	
	FS14	18285	1041	Intron 9	A	G	
	FS15	18727	1335	Exon 11	C	G	Synonymous (Val)
	FS16	18903	1336	Intron 11	A	G	
	FS17	19338	1337	3' UTR	A	G	
FADS2	FS18	7369	1404	5' end	A	G	
(FJ263685)	FS19	7376	1405	5' end	A	G	
	FS20	7468	1406	5' end	A	G	
	FS21	7498	1407	5' end	C	T	
	FS22	28505	1408	Intron 5	A	G	
	FS23	33876	1409	Intron 6	C	T	
	FS24	33924	1410	Intron 6	C	T	
	FS25	33958	1411	Intron 6	C	T	
	FS26	34075	1412	Intron 6	A	C	
	FS27	34076	1413	Intron 6	A	G	
	FS28	34149	1414	Intron 6	C	T	
	FS29	39965	1439	Intron 7	C	G	
	FS30	40048	1440	Exon 8	C	T	Synonymous (Phe)
	FS31	40155	1441	Intron 8	C	T	
	FS32	40181	1442	Intron 8	A	G	
	FS33	40232	1443	Intron 8	C	T	
	FS34	40235	1444	Intron 8	A	C	
	FS35	40240	1445	Intron 8	C	T	
	FS36	40271	1446	Intron 8	A	G	
	FS37	40279	1447	Intron 8	C	T	
	FS38	40281	1448	Intron 8	C	G	
FADS3	FS39	15879	2253	Intron 1	C	T	
(FJ263686)	FS40	15980	2254	Intron 1	C	T	
	FS41	16043	2255	Intron 1	A	G	
	FS42	16830	1828	Exon 3	C	T	Synonymous (Val)
	FS43	16884	1829	Exon 3	C	T	Synonymous (Ala)
	FS44	17228	1839	Intron 3	G	T	
	FS45	17379	1840	Exon 4	C	T	Synonymous (Phe)
	FS46	17463	1841	Intron 4	A	G	
	FS47	17469	1842	Intron 4	C	T	
	FS48	19386	1838	Intron 7	C	T	
SCD1	FS49	2894	1029	Intron 1	A	G	
(AY487830)	FS50	14416	1016	Intron 5	A	G	
	FS51	16663	1028	3' UTR	C	T	
	FS52	17800	1030	3' UTR	C	T	
SCD5	FS53	26896	1123	5' UTR	A	G	
(FJ263688)	FS54	27147	1124	Exon 1	A	G	Synonymous (Phe)
	FS55	27166	1125	Exon 1	C	T	Synonymous (Leu)
	FS56	147980	2178	Intron 3	A	G	
	FS57	148099	2179	Exon 4	C	T	Synonymous (Ile)
	FS58	148175	2180	Exon 4	A	G	Asn253Asp
	FS59	148243	2181	Intron 4	A	C	
	FS60	152501	2249	3' UTR	A	G	
	FS61	152626	2250	3' UTR	G	T	
	FS62	152657_9del		3' UTR	Ins(ACG)	Del	
	FS63	152708_9ins		3' UTR	Ins(TG)	Del	
	FS64	152917	2177	3' UTR	C	T	
	FS65	153384	2251	3' UTR	C	T	
	FS66	153403	2252	3' UTR	A	G	

Curriculum vitae

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