UNIVERSITÀ DEGLI STUDI DI SASSARI

DIPARTIMENTO DI SCIENZE ZOOTECNICHE

AGRIS – SARDEGNA

DIPARTIMENTO PER LA RICERCA NELLE PRODUZIONI ANIMALI

DOTTORATO DI RICERCA IN

SCIENZE E TECNOLOGIE ZOOTECNICHE

XXI CICLO

Association between ovine Stearoyl-CoA desaturase gene polymorphisms and the Conjugated Linoleic Acid content in sheep milk fat: results of a positional candidate gene study

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Acronymes

ACACA	:	acetyl-CoA carboxylase
AFLPs	:	mplified fragment length polymorphisms
AMY	:	alpha-amylase gene
BAC	:	bacterial artificial chromosome
BC	:	back cross
BCFA	:	branch-chain
CLA	:	conjugated linoleic acid
DGAT1	:	acylCoA-diacylglycerol-acyltransferase 1
DGAT1	:	acylCoA-diacylglycerol-acyltransferase 1
FA	:	fatty acids
FABP3	:	fatty acid-binding protein type 3
FAME	:	fatty acid methyl esters
FASN	:	fatty acid synthase
FASN	:	fatty acid synthase
GC	:	gas chromatography
GC-MS	:	gaschromatography mass spectrometry
GPAM	:	glycerol-3-phosphate acyltrans-ferase, mitochondrial
LIPA	:	lipase A, cholesterol esterase
LPL	:	lipoprotein lipase
MIR	:	mid-infrared
MS	:	mass spectrometry
MUFA	:	monounsaturated FA
MUTFA,	:	mono-unsaturated trans fatty acids
NF-Y	:	nuclear factor Y transcription factors
ORF	:	open reading region
PUFA	:	polyunsaturated fatty acids
QTL	:	quantitative trai loci
RA	:	rumenic acid
RAPDs	:	random amplified polymorphic DNA
RFLPs	:	restriction fragment length polymorphisms
SCD	:	stearoyl-CoA desaturase, Δ9-desaturase
SFA	:	saturated FA
SNPs	:	single nucleotide polymorphisms
SREBP	:	sterol response element binding protein
TAG	:	triacylglycerols
TFA	:	mono-unsaturated trans fatty acids
VA	:	vaccenic acid

1. Introduction

Sheep and goats were the first to be domesticated by early humans as farm livestock about 10,000 years ago. Originating from a few wild sheep and goat ancestor breeds in the region of today's Iraq, Iran, Syria and Eastern Turkey (Zeuner, 1963; Schaller, 1977; Zeder and Hesse, 2000; Hatziminaoglou and Boyazoglu, 2004, Haenlein et al 1984, Haenlein, 2007), sheep and goats were developed around the world into hundreds of different breeds totaling presently more than 1 billion sheep and 750 million goats (Park and Haenlein, 2006). The evolution of sheep and goats around the world and their role in agricultural development and human nutrition has been the concern of comprehensive studies in recent years in order to provide stimulus and support for further improvement, especially in developing countries (Glimp and Fitzhugh, 1977; CAST, 1982; King, 1988; IDF, 2000, 2005). Dairy goat and dairy sheep farming are a vital part of the national economy in many countries, especially in the Mediterranean and Middle East region (FAO, 2003), and are particularly well organized in Italy, France, Spain, and Greece (Park and Haenlein, 2006). Information on composition and physico-chemical characteristics of goat and sheep milk is essential for successful development of dairy sheep and goat industries as well as for the marketing the products. On the whole, Mediterranean dairy sheep systems have a dual purpose, with income from meat (milk-fed lambs) and milk, the milk being processed into high quality cheeses. Milk sales represent about 65 to 75 % of farm income, against 30% from meat sales. Thus, accounting also for the increase in consumption of sheep cheese and for the fact that most of the local breeds have a low to medium milk yield level, increasing milk production is still the most profitable objective for numerous breeds). Consequently, the breeding strategy corresponds usually to the dairy selection of the local breeds in their specific area and production systems for milk yield and composition (Barillet, 1997).

Most goat and sheep milk is processed into high quality cheese, often Protected Designation of Origin (PDO) cheese as laid down in the European Union legislation. Due to evolution of consumers' demand for typical cheese and for more quality and safety, dairy sheep and goats have to be improved

for production (milk yield) and milk composition (fat and protein) to remain competitive, but new functional traits related to reducing production costs and increasing product quality and safety must also be considered. These functional traits include resistance to diseases like mastitis, which also has implications for animal welfare. Increasing attention has been given for the last decade to functional traits, mainly on a quantitative genetics basis, but in recent years another window has been opened based on new molecular tools allowing the detection and mapping of genes of economic importance in farm animals. This development will provide new opportunities for marker assisted selection (MAS) or gene assisted selection (GAS) to be used in the selection for product quality and safety traits which are often difficult to measure (Barillet, 2007).

The Sardinian breed is the most important dairy breed in Italy, with about 60% of the Italian stock (8,166,978 head, ISTAT, 2005) which is prevalently reared in Sardinia.

The dairy sheep industry in Sardinia involves about 20,000 farms and three and half million ewes. Milk production, about 320,000 T per year, is entirely processed into different PDO cheeses.

The quality of dietary lipids could be an important modulator in terms of the morbidity and mortality of a lot of diseases like hyperlipidemia, arteriosclerosis, obesity, diabetes mellitus and hypertension; it is also assumed that one-third of human cancers is associated with dietary habits and lifestyle. Because of the rapid increase in the number of elderly people, these diseases are important medically and socio-economically. In this context the milk fat content and fatty acid composition (FA) play an important role. Thus, an increased interest has been paid on milk quality not only in terms of fat and protein contents (which only recently are being considered in the local milk payment system) but also in terms of nutritional value of the milk. FA composition influences milk fat quality, contributing to its physical (crystallization and fractionation of the fat, hardness and melting point of the butter), and sensory (free short chain FA, oxidation products) properties (Chilliard *et al.*, 2000). Short and medium chain saturated, branched, mono

and polyunsaturated and *cis* and *trans* conjugated FA in particular are potentially involved as positive or negative factors for human health (Hu *et al*., 1999).

Improving the nutritional quality of milk fat has been so the topic of recent research. Feed supplementation, the most popular way to improve the nutritional quality of milk, presents certain disadvantages. First, this approach ignores the animal genetic effect, second, this improvement is not permanent. Indeed, if supplementation is stopped, the additional nutritional quality disappears. The advantages of the genetic approach are linked to these disadvantages: genetic improvement is permanent and has the advantage of creating additional value through selection.

1.1. Basic composition of ruminant milk

Compositions of goat, sheep, cow and human milks are different (Table 1-1), but vary with diet, breed, individuals, parity, season, feeding, management, environmental (Moio et al., 1993) conditions, locality, stage of lactation, and health status of the udder (Parkash and Jenness, 1968; Schmidt, 1971; Linzell and Peaker, 1971; Larson and Smith, 1974; Posati and Orr, 1976; Underwood, 1977; Jenness, 1980; Haenlein and Caccese, 1984; Juàrez and Ramos, 1986; Park, 1991, 2006a).

Composition	Goat	Sheep	Cow	Human
Fat (%)	3.8	7.9	3.6	4.0
Solids-not-fat (%)	8.9	12.0	9.0	8.9
Lactose (%)	4.1	4.9	4.7	6.9
Protein (%)	3.4	6.2	3.2	1.2
Casein (%)	2.4	4.2	2.6	0.4
Albumin, globulin (%)	0.6	1.0	0.6	0.7
Non-protein N (%)	0.4	0.8	0.2	0.5
Ash (%)	0.8	0.9	0.7	0.3
Calories/100 ml	70	105	69	68

Table 1-1 Average composition of basic nutrients in goat, sheep, cow and human milk. Data from Posati and Orr (1976), Jenness (1980), Larson and Smith (1974).

Sheep milk contains higher total solids and major nutrient contents than goat and cow milk (Table 1-1).

1.2. Lipids in ruminant milk

1.2.1. General aspects of milk fat

Lipids are the most important components of milk in terms of cost, nutrition, physical and sensory characteristics that they impart to dairy products. Triacylglycerols (TAG) constitute the biggest group (nearly 98%), including a large number of esterified fatty acids. Consequently, TAG composition is very complex. Along with TAG, the lipid composition of ewe milk presents other simple lipids (diacylglycerols, monoacylglycerols, cholesterol esters), complex lipids (phospholipids) and liposoluble compounds (sterols, cholesterol esters, hydrocarbons) (Park, 2006a; Haenlein and Wendorff, 2006). Lipids are present in the form of globules, which in ewe are characteristically abundant in sizes less than 3.5 μm. Some studies found that the average fat globule size is smallest in sheep milk followed by goat milk (65% of globules less than 3 μm; Mens, 1985). This is advantageous for digestibility and a more efficient lipid metabolism compared with cow milk fat (Park, 1994). Structure and composition of the membrane is similar in the three species and represents approximately 1% of total milk fat volume (Scolozzi et al., 2003). The phospholipid profile is similar to that of the plasma membrane, which may confirm a common origin. This fraction accounts for roughly 0.8% of total lipids.

1.2.2. Fatty acids

Most fatty acid, from acetic (C2:0) to arachidic acid (C20:0), contain an even number of carbon atoms. Five fatty acids (C10:0, C14:0, C16:0, C18:0, and C18:1) account for >75% of total FA in sheep milk (Table 1-2). Levels of the metabolically valuable short and medium chain FA, caproic (C6:0, 2.9%) caprylic (C8:0, 2.6%), capric (C10:0, 7.8%), and lauric (C12:0, 4.4%) are significantly high in sheep (Alonso et al., 1999; Goudjil et al., 2004). These FA are associated with the characteristic flavours of cheeses and can also be used to detect admixtures of milk from different species. The most important factor between the intrinsic and extrinsic variables to modulate milk FA

composition is the feed, in particular adding lipid supplement to the diet as reviewed for sheep (Bocquier and Caja, 2001). To increase levels of polyunsaturated fatty acids (PUFA) in milk, dietary PUFA oil intake and factors which decrease their hydrogenation in the rumen have been successful for goat milk, such as trapping of FA in vegetable cells, high forage/concentrate ratios, and PUFA rich protected fat (Chilliard and Ferlay, 2004; Sanz Sampelayo et al., 2004). Chiofalo et al., 2004) found increases of monounsaturated fatty acids (MUFA) in milk after the administration of olive cake to ewe feed.

The main point of quantifying other minor milk lipid components, branch-chain (BCFA) and odd-number chain saturated fatty acids is that volatile BCFA lend characteristic flavours to many dairy foods. Their amounts in cheese are dependent on the composition of the milk fat substrate. BCFA concentration in sheep milk fat (2.0% of total FA; Goudjil et al., 2004) was made up of six different acids: iso-C14, iso- and anteiso- C15, iso- and anteiso-C17, and iso-C16, which are also predominant in bovine milk (Jensen, 2002).

FA contents of milk fat affect the technological properties and the nutritional value of dairy products. Sheep cheese is highly appreciated for its taste and flavour, but is commonly considered potentially negative for human health due to high content in fat matter. As a whole, the overall FA profile should be considered for its effects on human health. Data from sheep milk showed that the saturated FA (SFA) level in milk fat is quite high (more than 60 %) while monounsaturated FA (MUFA) and polyunsaturated FA (PUFA) are approximately 28% and 6% respectively (Cabiddu et al., 2005). A combination of milk lipids more favorable to human health would be around 30% saturated (Pascal, 1996), 60% monosaturated, and 10% polysaturated (Hayes and Khosla, 1992). The fatty acid profile of sheep's milk is therefore far from optimal. However, the observed variations in saturated, monounsaturated, and polyunsaturated suggest that the milk fat composition can be modified by various means (e.g., feeding and genetics) to be brought closer to the optimal profile (Palmquist et al., 1993). FA showed large

variability in terms of their repeatability estimates, with ranged from 14.1% for C18:3 to 57.3% for C4 (Carta et al, 2008). Many results are available describing the influence of feeding (Demeyer and Doreau, 1999; Chilliard et al., 2000); however, many fewer are available on breed and individual genetic differences, even though variation in the overall milk fat among breeds is well known (Interbull, 2005). Also, not all the fatty acids in a specific class (saturated, monounsaturated, or polysaturated) have the same effect on human health. In the case of saturated, although myristic acid is known for its negative effect on cardiovascular diseases, stearic acid does not seem to have this effect (Hu et al., 1999, Soyeurt et al, 2006a). Similarly, in polysaturated, the n-6 fatty acids appear to have negative effects on human health because of their overabundance in the Western diet. The current ratio of n-6:n-3 is estimated to be 15–20 to 1 in the human Western diet (Simopoulos, 2003). It is therefore important to check the global fatty acid profile in milk if one wants to assess the nutritional quality of sheep milk fat (Table 1-2).

1.2.2.1. Mono-unsaturated trans fatty acids

Mono-unsaturated trans fatty acids (MUTFA, TFA) content in milk fat ranges from 2.5% to 5% of total FA, depending on the species and the season. In general, sheep milk presented the highest quantities, after cow and finally goat milk fat. In recent years, TFA intake has been associated with the risk of coronary heart disease. The main daily source of TFA consumed by humans is partially hydrogenated vegetable fats and oils, although these compounds also occur naturally in ovine, caprine and bovine milk. Monoene TFA are the majority in all species. However, the isomer profile of hydrogenated vegetable fats is very different. During hydrogenation of vegetable fats a range of trans mono-unsaturated FA are principally formed (e.g. trans -9 C18:1, elaidic acid), while the main TFA in milk fat is trans-11 C18:1, vaccenic acid (IDF, 2005). The importance of vaccenic acid (VA) lies in its role as a precursor in the synthesis of the main isomer of the nutritionally valuable conjugated linoleic acid (CLA), cis-9 trans-11, which occurs in the

mammary gland (Griinari and Bauman, 1999) and also in some human tissues (Turpeinen et al., 2002).

Fatty acid	Sheep milk fat[a]		Goat milk fat[b]	
	Mean	*Mininum/maximum*	*Mean*	*Minimum/maximum*
C4:0	3.51	3.07–3.93	2.18	1.97–2.44
C6:0	2.90	2.68–3.44	2.39	2.03–2.70
C8:0	2.64	2.10–3.27	2.73	2.28–3.04
C10:0	7.82	5.54–9.73	9.97	8.85–11.0
C10:1	0.26	0.23–0.31	0.24	0.19–0.38
C12:0	4.38	3.48–4.92	4.99	3.87–6.18
C12:1	0.04	0.03–0.05	0.19	0.10–0.40
C13:0	0.17	0.13–0.22	0.15	0.06–0.28
C14:0	10.4	9.85–10.7	9.81	7.71–11.2
iso-C15:0	0.34	0.26–0.43	0.13	0.12–0.15
anteiso-C15:0	0.47	0.33–0.60	0.21	0.17–0.24
C14:1	0.28	0.19–0.50	0.18	0.17–0.20
C15:0	0.99	0.89–1.11	0.71	0.46–0.85
iso-C16:0	0.21	0.17–0.26	0.24	0.17–0.40
C16:0	25.9	22.5–28.2	28.2	23.2–34.8
iso-C17:0	0.53	0.44–0.59	0.35	0.24–0.52
anteiso-C17:0	0.30	0.26–0.36	0.42	0.30–0.50
C16:1	1.03	0.74–1.27	1.59	1.00–2.70
C17:0	0.63	0.58–0.70	0.72	0.52–0.90
C17:1	0.20	0.17–0.22	0.39	0.24–0.48
C18:0	9.57	8.51–11.0	8.88	5.77–13.2
C18:1 total	21.1	17.8–23.0	19.3	15.4–27.7
C18:2 total	3.21	2.89–3.57	3.19	2.49–4.34
C20:0	0.45	0.36–0.52	0.15	0.08–0.35
C18:3	0.80	0.52–1.04	0.42	0.19–0.87
C18:2 conjugate total	0.74	0.56–0.97	0.70	0.32–1.17

Table 1-2 Mean values and minimum and maximum contents of main fatty acids (% in total fatty acid methyl esters) in sheep and goat milk fat.
a Goudjil et al. (2004); b Alonso et al. (1999).

1.2.2.2. Triacylglycerols

The TAG structure of milk is responsible for the rheological properties of milk fat and its behavior during melting and crystallization. Their composition is of interest because it can be used to verify the origin of milk fat. TAG are almost invariably accompanied by small amounts of di- and mono-glycerides, mainly at position 1 and 2 molecules, probably being intermediates in the biosynthesis of TAG. The TAG of the three species present a wide range of molecular weights and chain lengths with carbon atom numbers beyond C26–C54. The TAG content in sheep milk has a bimodal distribution with a maximum at C36–C38 and C50–C52, and a minimum at C44–C46

(Partidario et al., 1998; Goudjil et al., 2003a). The chromatographic TAG profile of sheep milk shows similarities to that reported for cow milk (Precht, 1992). In goat milk the TAG content increased with the number of carbon atoms, reaching maximum (about 13% of total TAG) at C40–C42. Ewes and goats had a higher percentage of C26–C36 TAG than cow milk (24% versus 21%). These differences are related to the need for a TAG composition with appropriate melting points for fat secretion. Regarding the molecular species of TAG in goat and sheep milk using more than one chromatographic technique and mass spectrometry (MS) (Ruiz-Sala et al., 1996; Fontecha et al., 2000, 2005), it has been established, that in goat milk fat the most important TAG in quantitative terms are medium-chain FA (C8, C10, C12) and C18:1 as unsaturated FA (Fontecha et al., 2000). In sheep milk fat the primary TAG are composed of three fatty acids, C4, C16 and C18:1 (Najera et al., 1999; Fontecha et al., 2005), the same as in cow milk (Spanos et al., 1995; Fraga et al., 1998). Concentrations of TAG in which the FA C8, C10 or C12 were esterified was highest in goat > sheep > cow milk fat. The FA distribution in TAG in bovine milk is non-random (Christie, 1995), but there is no corresponding evidence for the distribution of FA in TAG in ovine or caprine milk. The comparison of theoretical and experimental values reveals a non-random distribution of the FA in the TAG of sheep and goat milk fat. TAG containing a short-chain FA, e.g., C4 or C6 are synthesized preferentially to TAG containing three medium- or three long-chain FA. This relates to the fact, that only one short-chain acid (C4 or C6) occurs per TAG molecule and stereospecific studies have shown, that short-chain FA in ruminant mammary glands are esterified only in position sn-3, whereas medium- and long-chain FA are esterified in all three glycerol positions.

1.2.2.3. Sterol fraction

Sterols are a minor fraction of total lipids in milk, the main sterol being cholesterol (300 mg/100 g fat, equivalent to 10 mg/100 ml cow milk). Small quantities of other sterols have been reported in cow milk (Jensen, 2002) including vegetable sterols (IDF, 1992). The sterol fraction of milk is of nutritional interest because high levels of cholesterol in plasma are

associated with an increasing risk of cardiovascular disease. Through analyses of the sterol fractions adulterant vegetable fats can be detected in milk and dairy products. Values reported for the cholesterol content of sheep and goat milk vary considerably due to different breeds and the use of different analytical techniques. Minor sterols represent 3–5% of the total sterol fraction.

1.2.2.4. Conjugated linoleic acid

Recently, research has focused on a group of FA named conjugated linoleic acid (CLA), which are present in milk and dairy products. There has been a surge of interest in CLA in man's diet because of increasing evidence, based largely on animal studies, suggesting potential benefits of CLA for man's health.

The generic name CLA is a collective term embracing all octadecadienoic acids (C18:2) with a conjugated double bond system in the 7–9, 8–10, 9–11, 10–12, 11–13 and 12–14 positions and a cis/cis, cis/trans, trans/cis and trans/trans geometrical configuration. The major isomer of CLA in natural food is cis-9 trans-11 (c9t11). The cis-9 trans-11 isomer is also the predominant isomer in meat from ruminants but constitutes less of the total (Chin et al., 1992; Shantha et al., 1994). The lower proportion of cis-9 trans-11 isomer in meat fat as compared to milk fat probably relates to effects of traditional high-concentrate, low-fiber diets fed to finishing cattle in the United States. The cis-9, trans-11 isomer was >90% of the total CLA in subcutaneous and intramuscular fat of German Simmental cattle fed corn-silage-based diets with a moderate level of grain supplement (Fritsche and Fritsche, 1998). From previous studies (Luna et al., 2005) it can be seen that most of the CLA in ewe milk corresponds to cis-9 trans-11 that represented more than 75% of total CLA; this percentage does not differ substantially from proportions reported for RA in total CLA in cow milk fat (approximately 80%; Sehat et al. 1998; Yurawecz et al. 1998, Khanal, 2004). Trans -11 trans -13, cis /plus trans /11–13, and cis /plus trans /7–9 were the main isomers after c9t11. Minor amounts of 8–10 and 10–12 C18:2 isomers were also found. The principal CLA dietary sources for humans are dairy products and

other foods derived from ruminant animals although CLA occurs naturally in many foodstuffs. Since current dietary recommendations include eating less animal fat, dietary CLA concentration is believed to have declined during the past 20 years. It is likely that the health benefits derived from CLA have therefore declined in relation to the decrease in consumption of animal fat. Thus there is an increasing interest in attempts to enrich the CLA content in milk, and so obtain dairy products with improved nutritional value.

1.2.2.5. Biological effects of CLA in health and disease

Hyperlipidemia, obesity, cancer, osteoporosis and diabetes are serious health problems plaguing the Western society during the aging process, which may be associated with dietary and lifestyle choices.

Data from animal models have been used to suggest that the cis-9 -trans 11 isomer (rumenic acid has been proposed as the common name for this specific CLA isomer by Kramer et al., 1998a) is responsible for CLA anticarcinogenic properties, as well as antiatherogenic effects (Khanal, 2004; Parodi, 2004; Lee et al., 2005b), whereas the trans-10 cis-12 CLA isomer has lean body massenhancing properties (Belury, 2002; Pariza, 2004).

A wealth of literature is available mainly from cell line and animal studies, comparatively, there is limited literature on the effects of CLA in human health. Research on the biological functions and health benefits of CLA dates back to the 1980s when Ha et al. 1987 made the seminal observation that CLA mixtures isolated from grilled beef, or from a base-catalyzed isomerization of linoleic acid, inhibited chemically induced skin neoplasia in mice. The antiobesity, antiatherogenic, and antidiabetic effects of CLA are supported by studies in animals, which led to the widespread use of CLA in the United States and Europe, especially among obese individuals (Bhattacharyaa et al 2006). Moreover, there are considerable variations between studies, and the dramatic beneficial effects seen in some animal models have not been reflected in human studies. This can be attributed to difference in dosage of CLA used in animal and clinical studies and differences in source of CLA (whether CLA was supplemented, in form of capsules or obtained from the diet). There are several indications that various

isoforms could have different biological actions, although most studies used a synthetically prepared CLA mixture. Commercial CLA supplements are isomeric mixtures, usually containing 2 major isomers, c9t11 and t10c12, in equal amounts. With the advent of technology, enriched or purified c9t11 and t10c12 CLA preparations have become commercially available in recent years, leading to studies examining the effects of these individual isomers in health-related disorders. Recent evidence suggests that c9t11 and t10c12 may have myriad effects in different biological systems. Indeed, it has been found that both the isomers exhibit significant biological activities, which often may be similar or opposite (Bhattacharya et al 2006).

1.2.2.6. Fatty acid content in ruminant milk

Most studies on ewe milk fat (Prandini et al., 2001; Barbosa et al., 2003) simply quantify the most prominent peak assigned to CLA as fatty acid methyl esters (FAME) by gas chromatography (GC) and shown that the GC peak can include more than one component and minor CLA isomers masked by the rumenic acid (RA) peak (Luna et al., 2005a). Moreover elucidation of individual CLA chemical structures in ewe milk fat has never been carried out. The isomer mixtures in milk are complex and no single technique can resolve all isomers. Different procedures have been used to elucidate CLA isomers in different foodstuffs. The majority of them have been quantified by silver-ion HPLC (Ag+ -HPLC) (Sehat et al.1998, 1999; Rickert et al. 1999; Adlof, 2003) but gaschromatography mass spectrometry (GC-MS) has been shown to be the most powerful tool for identifying them (Kramer et al. 2001; Roach et al. 2002; Dobson, 2003). A more complete qualitative and quantitative analysis involves applying both techniques, especially when GC is coupled to mass spectrometry (MS) to provide structural information. A detailed positional analysis of the double bonds of fatty acids can be performed subsequently using one of several fatty acid derivatives other than FAME, such as 4,4 dimethy-loxazoline (DMOX); this has already been applied to different food materials (Yurawecz et al. 1998; Roach, 1999, 2001). High-resolution MS with a selected ion-recording technique makes it possible to distinguish CLA from interfering fatty acids and discriminate

among the different isomers. The range of the relative composition of CLA isomers in ewe milk fat by silver-ion HPLC (Luna et al., 2005a) was similar to that reported in cow milk (Khanal, 2004).

Mid-infrared (MIR) spectrometry is an alternative to gas chromatography, with advantages such as a very high throughput (up to 500 samples/h; FOSS, 2005), ease of use, and availability (Soyeurt et al 2006). The infrared spectrum is caused by the absorptions of electromagnetic radiation at frequencies that are correlated to the vibrations of specific chemical bonds within a molecule (Coates, 2000). The spectrum therefore illustrates these absorptions at different wave numbers (cm−1) for a specific chemical composition (Smith, 1996). Mid-infrared spectrometry (400 to 4,000 cm−1) is particularly interesting because it is very highly sensitive to the chemical environment, as the fundamental absorptions of molecular vibrations occur in this region (Belton, 1997). Mid-infrared spectrometry can be used to estimate various traits quantitatively based on calibration equations. The estimation of fatty acid concentrations in milk and in milk fat using MIR spectrometry seems feasible. Although some fatty acids present in low concentrations in milk could not be predicted accurately (e.g., n-3 and 14:1cis-9), MIR spectrometry could predict most fatty acids (e.g., 14:0, 16:0, 18:0, 18:1, SAT, and MONO). Therefore, and because of the speed of analysis and the present application of the methodology in routine milk testing, MIR spectrometry is an important alternative in the dairy sector for providing indications of the fatty acid profiles in cow milk. The estimates based on MIR could be used as indicator traits for real underlying fatty acid concentrations, potentially in a multitrait setting using the appropriate selection index theory. Knowledge of these genetic values would open up opportunities for animal selection aimed at improving the nutritional quality of milk.

Total CLA mean content seems to decrease in the following order: ewe > cow> goat milk fat, 1.08%, 1.01% and 0.65%, respectively (Jahreis et al., 1999), but information about small ruminants is too scant to offer a full picture. Milk CLA concentration in different ruminant species varied with the season mainly due to variations in feeding factors (Chilliard and Ferlay,

2004). The greatest seasonal differences were measured in sheep milk, 1.28% in summer and 0.54% at the end of the winter period (Jahreis et al., 1999). Milk fat has been reported to contain not only the highest level of the beneficial CLA, but also the highest content of VA (physiological precursor of CLA) (Jahreis et al., 1999).

CLA content in dairy products is affected by many factors, animal feeding strategies and specifically diets with oil seed or oil supplements rich in PUFA have been effective in enriching milk of the three dairy species (Stanton et al., 2003; Khanal and Olson, 2004; Chilliard and Ferlay, 2004), especially of dairy goats (Chilliard et al., 2004) and dairy ewes (Luna et al., 2005). Effects of feeding fresh forages and the seasonal changes of Mediterranean natural pastures on FA, especially on CLA composition and its precursors in sheep milk have been reported (Addis et al., 2006; Cabiddu et al., 2005; Nudda et al., 2005). Enhancing CLA content by dietary changes results also in lower proportions of saturated FA and greater amounts of mono-unsaturated FA (including VA) and PUFA. Generally, free oils are more effective than whole or treated oilseeds (Chilliard et al., 2002). In ewes fed a linseed supplement no changes were observed in milk yield and protein content, but the fat content decreased slightly throughout lactation (Luna et al., 2005).q In ewes fed olive cake, a positive effect on milk yield and no changes in milk composition were reported (Chiofalo et al., 2004). Previous studies in cow (Kelsey et al. 2003) and ewe (Barbosa et al. 2003) milk fat only observed little variation in CLA content among breeds fed the same diet. Dietary factors, however, are known to greatly affect CLA in milk fat. Such factors include, foods that alter the rumen environment and thereby affect the bacteria responsible for biohydrogenation, and the lipid substrates consumed.

1.2.2.7. Biosynthesis of CLA

The CLA found in milk and meat fat of ruminants originate from two sources (Griinari and Bauman, 1999). One source is CLA formed during ruminal biohydrogenation of linoleic acid. The second source is CLA synthesized by the animal's tissues from *trans*-11 C18:1, another intermediate in the

biohydrogenation of unsaturated fatty acids. Thus, the uniqueness of CLA in food products derived from ruminants relates to the incomplete biohydrogenation of dietary unsaturated fatty acids in the rumen. Ironically, rumen biohydrogenation of dietary lipids is responsible for the high levels of saturated fatty acids in fat of ruminants, a feature considered undesirable for some aspects of human health, as well as for ruminant fat containing CLA, fatty acids with many putative beneficial effects on human health.

1.2.2.8. Rumen Biohydrogenation

The lipid composition of forages consists largely of glycolipids and phospholipids, and the major fatty acids are the unsaturated fatty acids linolenic (C18:3) and linoleic (C18:2) acid. In contrast, the lipid composition of seed oils used in concentrate feedstuffs is predominantly triglycerides containing linoleic and oleic acid (*cis*-9 C18:1) as the predominant fatty acids. When consumed by ruminant animals, dietary lipids undergo two important transformations in the rumen (Dawson and Kemp, 1970; Keeney, 1970; Dawson et al., 1977). The initial transformation is hydrolysis of the ester linkages catalyzed by microbial lipases. This step is a prerequisite for the second transformation: biohydrogenation of the unsaturated fatty acids. Bacteria are largely responsible for biohydrogenation of unsaturated fatty acids in the rumen; protozoa seem to be of only minor importance (Harfoot and Hazlewood, 1988). For a number of years, the only bacterium known to be capable of biohydrogenation was *Butyrivibrio fibrisolvens* (Kepler et al., 1966). However, as research efforts expanded, a diverse range of rumen bacteria have been isolated that have the capacity to biohydrogenate unsaturated fatty acids. Biohydrogenation of unsaturated fatty acids involves several biochemical steps and investigations with pure cultures suggest that no single species of rumen bacteria catalyzes the complete biohydrogenation sequence. Kemp and Lander (1984) divided bacteria into two groups based on the reactions and end products of biohydrogenation. Group A bacteria were able to hydrogenate linoleic acid and α-linolenic acid, *trans*-11 C18:1

being their major end product. Group B bacteria utilized *trans*-11 C18:1 as one of the main substrates with stearic acid being the end product.

The rumen is the site of intense microbial lipid metabolism. Lipolysis of dietary glycolipids, phospholipids and triacylglycerol releases free fatty acids, which are hydrogenated to a large extent. The amount of conjugated dienoic acids in cows' milk and butter has been correlated positively with dietary intake of linoleic acid, indicating that CLA formed in the rumen is incorporated into milk fat. The extent of rumen biohydrogenation mainly depends on the type of diet. This has been shown to be due to a drop in pH, limiting at first lipolysis, and thus hydrogenation, which occurs only on free fatty acids. A large amount of dietary linoleic acid and a decrease in the rate of hydrogenation are the two main factors that contribute to an increase in the concentration of the intermediate compounds CLA and *trans* monounsaturated fatty acids.

A portion of CLA formed in the rumen by biohydrogenation of linoleic acid, escapes further biohydrogenation and is absorbed in the digestive tract (Griinari and Bauman, 1999). The extent of this process is minimal, while the intermediate product of CLA biohydrogenation, vaccenic acid, accumulates (Harfoot and Hazelwood, 1988).

1.2.2.9. Tissue Synthesis of CLA

The other important source of vaccenic acid (VA) in the rumen is the biohydrogenation of the linolenic acid (C18:3). This pathway does not have as intermediate CLA. VA produced in the rumen is desaturated to produce CLA in the mammary gland tissues by Stearoyl-CoA desaturase (SCD, Δ9-desaturase). There is evidence that milk CLA basically comes from mammary synthesis by the action of SCD on VA provided by the rumen. Large variations in CLA content of milk have been reported. Typical concentrations of CLA in milk fat are 3–6 g/kg fat, but CLA concentration in milk can vary widely among herds. Animal variation in the CLA content of milk was high, affecting the CLA content of milk almost as much as the diet offered but thus differences in ruminant species (Jarheis *et al*., 1997), cattle breeds (Lawless *et al*., 1998) and between individual cows (Kelly *et al*., 1998)

can probably be explained by the different activities of SCD in the mammary gland, even when they were fed with the same diet.

1.2.3. Encoding genes for enzymes directly involved in fatty acid metabolism

Stearoyl-CoA desaturase is a key enzyme in the cellular biosynthesis of monounsaturated fatty acids. Because it plays an essential role in the lipid metabolism, SCD gene was hypothesized as candidate gene of the quality of milk fat.

Acetyl-CoA carboxylase (ACACA), is the flux-determining enzyme in the regulation of fatty acid synthesis in animal tissues. Different expression of this gene in the lactating mammary gland (Veltri, 2000) of two Italian sheep breeds (Gentile di Puglia and Sarda) suggested a direct involvement of this gene in the fatty acid synthesis during lactation, because of the different milk fat content of the two breeds. Single nucleotide polymorphisms (SNPs) were detected both in Promoter I (Moioli et al., 2005a) and Promoter II (Moioli et al., 2005b) of this gene SNPs frequency is significantly different between the Sarda breed and three other Italian sheep breeds (Gentile di Puglia, Comisana and Sopravissana). However, the effect of the SNPs on the ACACA gene expression has not yet been investigated.

Fatty acid synthase (FASN) is a multifunctional protein that carries out the synthesis of fatty acids so it plays a central role in de novo lipogenesis in mammals. Roy et al. 2006 defined the genetic structure and expression of the bovine FASN gene. Mapping studies placed FASN on BTA19 (19q22) where several quantitative trait loci (QTL) affecting milk-fat content and related traits have been described (Roy et al., 2006). Recentely SNPs associated with fatty acid composition of longissimus dorsi muscle of Angus bulls were identified in the exons that encode for the thioesterase domain of the bovine fatty acid synthase (FASN) gene (Zhang et al. 2008).

The lipoprotein lipase (LPL) is responsible for the hydrolyzation of triglycerides to glycerol and free fatty acids. It is synthesized in the epithelial cells of the mammary gland, and influences the release of fatty acids in the

mammary tissue. The differences in the gene expression of LPL in the lactating mammary gland of two breeds of sheep (Sarda and Gentile di Puglia) were studied by Graziano et al. (2005b). Consistent differences in the gene expression between the two breeds, at different lactation stages, were found but structural analysis of the gene will still be performed to assess the cause of the different gene expressions (Moioli et al 2007).

The acylCoA-diacylglycerol-acyltransferase 1 (DGAT1) is considered to be the key enzyme in controlling the synthesis of triglycerides in adipocytes. In cattle a polymorphism or a mutation in this gene is strongly associated with milk yield and composition (Grisart et al., 2004). In sheep DGAT1 is an obvious candidate gene for milk fat content by the fact, that a QTL was detected in chromosome 9, which is homologous to the bovine chromosome 14 region (Barillet et al., 2005).

1.2.3.1. Other putative candidate genes

Fatty acid-binding proteins (FABPs) play key roles as transport vehicles of fatty compounds throughout the cytoplasm. The ovine fatty acid-binding protein type 3 (FABP3) gene has been chosen as a functional candidate gene for milk traits (Calvo et al., 2002). Complete sheep FABP3 gene was sequenced and revealed 13 SNPs. In addition, two SNPs were screened in different sheep breeds, and have been tested in a daughter design comprising 13 families. No association was found between estimated breeding values for milk yield, protein and fat contents and genotypes across families; however, in withinfamily analysis, one family showed a significant effect for fat contents. These results could indicate linkage disequilibrium between the FABP3 gene and a quantitative trait locus for fat contents, with the heterozygous genotype associated with a positive effect in this trait (Calvo et al., 2004a).

On the basis of comparisons between cattle and sheep genome mapping information, the ovine alpha-amylase gene (AMY) was examined as a possible genetic marker for milk traits in sheep (Calvo et al., 2004b). DNA fragments corresponding to two different AMY genes were isolated, and one SNP in intron 3 and one GTG deletion in exon 3 of the 2360 bp DNA

fragment were found. No association was found between estimated breeding values for milk yield, protein and fat contents and AMY genotypes in a daughter design comprising 13 Manchega families with an average of 29 daughters per sire (Calvo et al., 2004b).

Fine mapping and association analysis with milk traits of an ovine region (chromosome 1), related to quantitative trait loci on bovine, revealed the following putative candidate gene for milk traits: cingulin (CGN) gene (Calvo et al., 2005); lysophosphatidic acid phosphatase protein (ACP6) gene (Calvo et al., 2005); annexin A9 protein (ANXA9) gene (Calvo et al., 2005); alpha amylase isoform 2 gene (Calvo et al., 2005).

1.2.3.2. Stearoyl-CoA desaturase

Mammalian stearoyl-CoA desaturase belongs to a family of desaturases that has been highly conserved throughout evolution in animals, plants, and yeast. SCD is a microsomal enzyme that in conjunction with nicotinamide adrenine dinucleotide, the flavoprotein cytochrome b5 reductase and the electron acceptor cytochrome b5, in the presence of molecular oxygen, introduces a single double bond in a spectrum of methylene-interrupted fatty acyl-CoA substrates. The SCD protein is an integrated endoplasmic reticulum membrane protein. It comprised of over 62% non-polar amino acid residues and is largely embedded in the endoplasmic reticulum membrane, with active sites exposed to the cytosol. A site-directed mutagenesis study confirmed that clusters of histidine-rich motifs are required for catalytic activity (Heinemann et al. 2003.).

Because it plays an essential role in the lipid metabolism, SCD gene was hypothesized as candidate gene of the quality of milk fat. SCD can use different fatty acids as substrate and influences the amount of several unsaturated fatty acids. The preferred substrates for SCD are palmitoyl- and stearoyl-CoA, which are converted to palmitoleoyl- and oleoyl-CoA, respectively. These products are the most abundant monounsaturated fatty acids in the various kinds of lipids, including phospholipids, triglycerides, cholesteryl esters, wax estes, and alkyldiacyloglicerols.

SCD mRNA and gene

The genes encoding SCD have been cloned and characterized in a number of mammalian species including rodents, ovine, porcine, bovine and human (Ward et al. 1998; Zhang et al. 1999; Bernard et al. 2001; Miyazaki et al. 2003).The cluster of SCD genes has been assigned to mouse chromosome 19 and it is overlapped by an identified QTL which is associated with elevated plasma levels of free fatty acids and triglycerides. Human SCD gene is located in HSA10, and a processed pseudogene in chromosome 17 has been identified (Zhang et al., 1999). In ruminant species, SCD genes have been mapped to ovine chromosome 22q21 and bovine and caprine chromosomes 26q21 (Campbell et al., 2001; Bernard et al., 2001).

The bovine SCD gene just like its caprine counterpart, comprises six exons and five introns and contains an open reading frame of 1080 nucleotides coding for a protein of 359 amino acids residues (GenBank Accession N°. AY241932 Bos taurus gene and AY241933 Bos taurus complete cds; AH011188 Capra hircus gene.), with a high level of similarity (more than 94%) between the three species. At the levels of nucleotide and amino acid sequences, the SCD gene family shows a high level of identity of the open reading frame among the orthologous genes and presents a conserved amino acid residues (and their spacing) involved in the catalysis of the Δ9 desaturation reaction (Shanklin et al., 1994; Zhang et al., 2001). The sheep sequence differs from the goat for one SNP in exon 4, giving rise to a different amino acid codon. Similarity of the sheep and goat gene with the cattle gene is very high, being two codons in exon 6 the only difference. However, Graziano et al. (2005b), who compared the gene sequence of five animals of the Sarda breed with the published sequence (AJ001048) found evidence of four SNPs in all five animals, causing an amino acid codon change, as well as one polymorphism in one individual only.

The promoter regions of the human (Bene et al. 2001; Zhang et al. 2001), chicken (Lefevre et al. 2001), and mouse (Ntambi et al. 1988; Kaestner et al. 1989; Mihara 1990) SCD genes have been isolated, cloned, and

characterized. It has been shown in these studies that there is a conserved PUFA response region in all three, and that this includes critical binding sites for sterol response element binding protein (SREBP) and Nuclear factor Y (NF-Y) transcription factors (Keating et al.2006). The human promoter study also indicated that there are two different transcription start sites, and the transcription initiation at these sites may be dependent on tissue-specific factors (Zhang et al. 2001).

The SCD cDNA sequence from human, mouse, rat, and goat contain unusually long 3´ untranslated regions (3´-UTR) that are derived from single exons. The role of such a long 3´- noncoding stretch is currently unknown but it may influence the mRNA stability and thereby plays a role in the regulation of expression of SCD genes (Jackson 1993). In human, the 3´-UTR region contain two functional polyadenylation signals resulting in two mRNAs transcripts (of 3.9 and 5.2 Kb) that are differentially expressed in different tissues (Zhang et al., 1999).

SCD isoforms and gene expression

The major difference between species is the variable number of SCD genes.

Many mammalian genomes contain in fact multiple stearoyl-CoA desaturase isoforms. The physiologic significance of this is currently unknown but could be related to the lipid metabolism characteristics in each specie. To date, four SCD isoforms have been described in mice (SCD1, SCD2, SCD3, and SCD4) (Kaestner et al. 1989, Miyazaki et al.2003, Ntambi et al. 1988, Zheng et al. 2001), three isoforms have been found in hamster (SCD1, SCD2 and SCD3) (Wang et al. 2008) and two isoforms have been described in rat (SCD1 and SCD2), in humans (SCD1 and SCD5) (Wang et al.2005, Zhang et al. 1999), in cattle (SCD1 and SCD5) (Chung et al. 2000), in sheep (SCD1 and SCD5) (Lengi et a. 2008), in pig (SCD1 and SCD5) (Lengi et a. 2008) and in chicken (SCD1 and SCD5) (Lengi et al,. 2008).

While the reason for the existence of multiple isoforms in some species is unclear, there is evidence for divergent tissue-specific expression of different isoforms. The four mice isoforms displayed similar desaturation activities

towards stearoyl-CoA and palmitoyl-CoA but have different tissue distributions. The expression of rodent SCD1 is sensitive to dietary manipulations, being highly induced under fat-restricted diets, and rapidly down regulated by diets high in poly-unsaturated fatty acids and regulation is facilitated by its short half-life. In the mouse, SCD1 is expressed predominantly in lipogenic tissues (Ntambi et al.1988), SCD2 is preferentially expressed in brain and neuronal tissues (Kaestner et al. 1989), SCD3 is expressed in sebocytes, preputial gland and Harderian gland (Zheng et al. 2001), and SCD4 expression is restricted to the heart (Miyazaki et al. 2003). In humans, SCD1 is most highly expressed in adipose tissue and liver while the second SCD gene, termed SCD5, is highly expressed in brain and pancreas (Wang et al. 2005). In addition to tissue specific expression, there is evidence to suggest that some SCD isoforms may differ in their preferred substrate specificity. For example, murine SCD1, SCD2 and SCD4 have been shown to desaturate both palmitoyl-CoA and stearoyl-CoA, while murine SCD3 desaturates palmitoyl-CoA but not stearoyl-CoA (Miyazaki et al., 2006). Nucleotide sequence comparison of the four mouse SCD cDNAs revealed that the homologous region is limited only to the protein coding sequence (more than 88% identity), with no significant homology in either the 5´or the 3' noncoding regions. The tissue and substrate specificity of SCD isoforms suggests that each may have a distinct physiological role. Although the mouse and human genomes both contain multiple SCD isoforms, there appear to be differences in the evolutionary origins of these genes. In the mouse, all four SCD isoforms are contained within a 200 kb region of chromosome 19 and appear to have arisen from tandem gene duplication events (Miyazaki et al. 2003). In the human, the SCD1 and SCD5 genes are on separate chromosomes, suggesting that they are not the result of a similar gene duplication event. Furthermore, while the human SCD1 gene appears to be an ortholog of mouse SCD1, SCD5 appears to be a distinct stearoyl-CoA desaturase gene rather than an ortholog of any of the mouse SCD isoforms (Wang et al. 2005) or of any other mammalian SCD gene but displays a tissue distribution pattern similar to mouse SCD2 (Wang et al.

2005, Beiraghi et al. 2003). SCD 5 has also been found in chimpanzees and orangutans and was originally thought to be a primate species by a single gene duplication event occurring after the divergence of primates from other mammalian species (Wang et al. 2005). However Lengi et al 2007 described an SCD 5 homolog in cattle and recently also in pigs, sheep and chickens rising by an early gene duplication event (Lengi and Corl 2008). The finding of SCD 5 homologs in both mammalian and non-mammalian vertebrate specie suggests that this gene may be much more widely existent than originally thought.

The ruminant SCD5 sequences (bovine and ovine) show a very close evolutionary relationship to each other, as do the primate SCD5 sequences (human and orangutan). It is known that human and bovine SCD5 genes are located on separated chromosome from SCD1, and that the SCD5 structure in bovines and humans (5 exons and 4 introns) (Zhang et al. 2005, Lengi et al. 2007) differs from the highly conserved 6 exons, 5 introns structure of the bovine, caprine (Bernard 2001), porcine (Ren et al. 2004), chicken and human SCD1 genes as well as all 4 murine SCD genes. This data support the hypothesis that SCD5 is a distinct desaturase gene that existed before the divergence of mammals but was lost during the evolution of at least some rodent species. It is not clear why rodents have evolved four SCD genes without SCD5 isoform. It is possible that later gene duplication events during the evolution of rodents, leading to the creation of additional SCD1 homologs, allowed these species to compensate for and survive the loss of SCD5.

The ovine SCD1 amino acid sequence has 89–93% identity with mouse, rat and human SCD1s (Ward et al.1998). However, it is not possible to ascertain whether the ovine SCD1 has greater identity with mouse SCD-1 or with mouse SCD-2, as both appear to diverge approximately equally from the ovine SCD (89% identity).

SCD expression is modulated by dietary factors (e.g. PUFA, cholesterol, vitamin A), hormonal signals (e.g. insulin, glucagon), environmental factors (temperature changes, metals, alcohol, thiazolinediones), peroxisomal

proliferators, and developmental processes. Most of these factors have been shown to regulate SCD expression/activity in liver and adipose tissue, however some of them control also muscle SCD. There are species differences in the tissue distribution of Δ9-desaturase. For rodents, concentrations of mRNA and enzyme activity are greatest in liver (Ntambi et al.1995). In contrast, growing sheep and cattle have substantially greater Δ9-desaturase in adipose tissue, as indicated by mRNA abundance and enzyme activity. Thus, adipose tissue seems to be a major site of endogenous synthesis of *cis*-9, *trans*-11 CLA in growing ruminants. The mammary gland is the apparent site of endogenous synthesis of *cis*-9, *trans*-11 CLA for lactating ruminants, based on the activity of Δ9- desaturase (Kinsella et al.1972). In vivo results are also consistent with the lactating mammary gland being of primary importance in endogenous synthesis of *cis*-9, *trans*-11 CLA during lactation. Ward et al. 1998 determined tissue-specific changes in mRNA abundance of Δ9-desaturase in sheep at different physiological states and observed a decrease in mRNA abundance in adipose tissue and an increase in mammary tissue with the onset of lactation. Consistent differences in the gene expression of SCD in the lactating mammary gland of two breeds of sheep (Sarda and Gentile di Puglia) were reported by Graziano et al. (2003a, 2005a).

SCD Polymorphisms

The mammalian SCD gene, isolated in several species including mouse, cattle and human, shows a conserved genomic structure, spanning approximately 15–24 kb and consisting of six exons and five introns.

It has been hypothesized that diet-independent variations in CLA content of milk are caused by differences in mammary SCD activity, associated with either regulation of expression and polymorphism of SCD gene, or post-translational regulation of the SCD enzyme (Peterson et al. 2002).

Till now, no polymorphism has been detected in the promoter region in the breeds and groups of animals which are characterized by considerable differences in milk CLA content (Keating et al. 2005). Taniguchi et al. 2004

have been detected eight single nucleotide polymorphisms in the full-length SCD cDNA in the Japanese Black cattle breed, highly appreciated for its marbled beef and low fat melting point. Three of this SNPs have been detected in the fifth exon, were in complete linkage disequilibrium and resulted in 2 haplotypes. One of these, 878 T-C, causes a substitution valine (allele V) with alanine (allele A) in SCD protein and has been related to higher level of SCD activity and monounsaturated fatty acids concentration in milk and to higher MUFA percentage and lower melting point in intramuscular fat (Taniguchi et al., 2004). Because valine is highly conserved across mammals, it is considered the ancestral amino acid in that position (Taniguchi et al., 2004).

A recent study highlighted a relationship between SCD gene polymorphism and SCD activity and fatty acid composition also in Italian Friesian cows (Conte et al. 2006). The same SCD gene polymorphism has been related to fatty acid composition of beef in the Japanese Black cattle breed, (Taniguchi et al. 2004) and also the genotype distribution was similar with the data reported in Taniguchi et al. study: haplotype “A” allele frequency was major than haplotype “V” one. SCD polymorphism showed a significantly relationship with mammary gland desaturation activity. Results of Mele et al. 2007 provide some indication of an association between SCD locus and the fatty acid profile also in sample of Italian Holsteins. Frequencies of SCD genotypes observed were in agreement with results reported by the above-mentioned study Taniguchi et al 2004: higher frequency of the “A” allele against “V” allele. In particular, associations of SCD genotype with MUFA content and the C14:1/C14 ratio, an indicator of mammary desaturase activity, were indicated.

Kgwatalala et al 2007 found SNPs in the open reading frame (ORF) of the SCD gene in Canadian Holstein and Jerseys cattle. They found a total of 4 and 3 SNPs in Canadian Holsteins and Jerseys, respectively. Based on GenBank AY241933, the SNPs were found at 702 (A–G), 762 (T–C) and 878 (C–T) nucleotide positions of the SCD gene in both Holsteins and Jerseys and an additional SNP at 435 (G–A) was unique to Holsteins. All the reported

SNPs led to transitional exchanges (A/G or C/T) and three of them (435 G-A, 702 A-G and 762 T-C) were synonyms leading to no amino acid change in the resulting protein. The SNP at nucleotide 878 (C-T) was however predicted to cause amino acid substitution in the resulting SCD protein, valine/alanine. Synonymous mutations in the ORF regions of most genes are common in order to introduce genetic variability and at the same time maintain the integrity and functionality of the resulting proteins (enzymes) and minimize disturbances to important physiological processes catalyzed by the enzymes. Multiple comparisons of the SCD amino acid sequences from different mammalian species show a highly conserved region in the two-third carboxylterminal part of the enzyme suggesting that this may contain the catalytic site (Shanklin et al. 1994; Bernard et al. 2001), and the occurrence of a nonsynonymous SNP (878 C-T) outside this region and only synonymous SNPs within this region are consistent with this hypothesis. SNPs 702 A-G, 762 T-C and 878 C-T are consistent with those found in the Japanese Black cattle (Taniguchi et al. 2004) whereas SNP 435 G-A has reported only in Holstein (Kgwatalala et al 2007). This SNPs in the ORF of the SCD gene characterize four different genetic variants which results in two protein variants. The two genetic variants (A and B) found in both Jersey and Holstein breeds have been previously described (Taniguchi et al. 2004) while the other two variants (A1 and B1) found only in Holsteins are being described for the first time by Kgwatalala et al 2007. This studies demonstrated nucleotide differences between the SCD genes of the Canadian Holstein and Jersey breeds, which may account for the differences observed between the breeds in the levels of CLA and MUFA in their milks.

Milanesi et al (2008) recently reported evidences for the direct influence of SCD polymorphism on fatty acid composition of milk and beef. They showed that Italian cattle breeds display a high variability in exon 5 of SCD gene and described three new haplotypes, one of these characterizing specifically indigenous beef breeds, moreover they found a significant partition of haplotype variability between dairy and beef purpose breeds. In dairy cattle they observed lower haplotype variability and the highest value of the 878C

allele. However, in beef breeds they observed high number of haplotypes and a lower frequency of the 878C allele, with the exception of Chianina, an Italian beef cattle belonging to a group of breeds particularly appreciated for meat quality certified by the first IGP quality trademark officially approved for meat by the European Union Community. In this breed the frequency of the C allele is close to the frequency observed in the dairy breeds (Grey Alpine, and It. Brown). These observations may reflect a differential selective pressure on SCD polymorphism or a signature of a different evolutionary history of this group of breeds, recently hypothesized by the study of Pellecchia et al. (2007).

Macciotta et al (2008), hypothesizing that a gene involved in energetic pathways may affect other productive traits as milk, fat, and protein yields, investigated the relationships between the SNP polymorphism located in exon 5 of the SCD locus (Taniguchi et al., 2004) and milk production traits in Italian Holstein cattle. They showed an association between the polymorphism at exon 5 of the SCD locus and daily milk and protein yields in a sample of Italian Holsteins.

Further association studies are needed to better evaluate the role of these polymorphisms in fatty acid composition, particularly in CLA content, in ruminant-derived food products.

1.3. Genetic improvement of milk quality

The goal of improving milk quality is not easily achievable since the concept of quality involves many aspects. Milk can be evaluated for its safety, constituents content, technological and organoleptic characteristics, nutritional, and functional properties. All these features are, to different extents, under genetic control (Pilla et al., 2006). Unfortunately, sheep and goat milk constituents have been little taken into account in the selection programmes of small ruminants for two main reasons: the first is that the collection of a representative sample at each monthly record and the analysis costs might not be paid back in the low input production systems, as the ones

where sheep and goats are mostly reared. The regulations of the International Committee for Animal Recording (ICAR) do not request compulsory analysis for milk composition either in sheep or goats, for the above mentioned problems. However, in some countries, for some breeds, the analyses of fat and protein content in milk are regularly performed (Astruc et al., 2005): France, Germany, Italy (only Sarda breed), Spain (Churra, Castellana, Manchega, Laxta breeds), Switzerland, USA, Canada, Australia and these traits have been taken into account in some breeding programmes such as for the Lacaune and Pyrenean breeds, in France, and for Manchega and Churra breeds in Spain. Estimates of their heritability are highly variable, depending on the breed and the used sampling system. Barillet et al. (1998) reviewed the genetic variation of dairy traits for small dairy ruminants, and concluded that differences in the estimates of heritabilities depend on the sampled animals and also on the employed statistical procedure, either lactation records or test-day records.

Recently, researchers have focused also on milk faty acid profile, because of the increased interest in lipids and their effects, both on human health, and on the taste of the products (Moioli et al 2007). Nevertheless only few studies are available on the genetic determinism of these traits. This is mainly due to the fact that large-scale phenotyping is needed to produce accurate genetic studies and, that until recently, the only available technique has been very time-consuming and costly gas chromatography technique. However, Soyeurt et al. (2006a) showed that genetic variation exists across and within cattle breeds for FA profile of milk, using a mid-infrared spectrometry technique which was less laborious and expensive (Soyeurt et al. 2006b). Recent results showing that bovine milk fatty acid composition is determined to a large extent by genetics, indicate that selective breeding can be an effective means to alter the composition of milk fat in this species (Schennink et al., 2008; Soyeurt et al., 2007). The difficulty of a large-scale phenotyping is even more evident in sheep where the individual recording of any trait is much more costly compared to dairy cattle due to the low individual income from each ewe. However, substantial advances have been made over the

past decades through the application of molecular genetics in the identification of loci and chromosomal regions that contain loci that affect traits of importance in livestock production (Andersson et al. 2005). This has enabled opportunities to enhance genetic improvement programs in livestock by direct selection on genes or genomic regions that affect economic traits through marker-assisted selection and gene-assisted selection (Dekkers and Hospital 2002).

1.3.1. Potential benefits of MAS

The potential benefits of using markers linked to genes of interest in breeding programmes, thus moving from phenotype based towards genotype-based selection, have been obvious for many decades. However, realization of this potential has been limited by the lack of markers. With the advent of DNA-based genetic markers in the late 1970s, the situation changed and researchers could, for the first time, begin to identify large numbers of markers dispersed throughout the genome of any species of interest and use the markers to detect associations with traits of interest, thus allowing MAS finally to become a reality.

However, despite the considerable resources that have been invested in this field and despite the enormous potential it still represents, with few exceptions, MAS has not yet delivered its expected benefits in commercial breeding programmes for crops, livestock, forest trees or farmed fish in the developed world. Evaluating the potential merits of applying MAS as a tool for genetic improvement, some of the issues that should be considered are its economic costs and benefits, its potential benefits compared with conventional breeding or with application of other biotechnologies, and the potential impact of intellectual property rights on the development and application of MAS.

The benefits of marker-assisted selection (MAS) to breeding programmes depend on a number of conditions that are relevant for most breeding programmes across species. These conditions include the existence of a genotype test predicting phenotypic differences, the economic value of these

differences and the value of the genotypic information within the breeding programme. The value of genetic information will depend heavily on the socio-economic context of the breeding programme and the production system. In a technical sense, the value of this information is basically driven by the increase in selection accuracy resulting from knowledge of genotypes, which in turn will differ between animals from different age classes. In particular, the relative increase in selection accuracy of the youngest selection candidates will be critical to the value of MAS. However, technical arguments about increased selection accuracy are of little value if these selection criteria are poorly developed or accepted within the production system. The application of new technologies such as MAS in animal breeding programmes therefore depends not only on a number of technical aspects associated with increased rates of genetic improvement, but also on the commercial structures of the industry.

MAS is most useful for traits that cannot be improved easily by phenotypic selection, either because they are difficult to measure on young animals (before sexual reproduction), or because of low heritability.

Dairy sheep are predominantly found in the Mediterranean region with both milk and meat production being economically relevant traits to farmers. A great variety of breeds are being targeted in selection programmes for the improvement of milk yield and milk composition but the importance of functional traits such as udder characteristics and mastitis susceptibility is increasing (Barillet, 1997). Genetic improvement for either for dairy traits, being sex-limited and measured after the first offspring are born, and milk fat components, which are so costly to measure, would particularly benefit from MAS.

1.3.2. Molecular Markers

Application of molecular genetics using marker-assisted selection (MAS) for genetic improvement relies on the ability to genotype individuals for specific genetic loci. Molecular marker maps, the necessary framework for any MAS programme, have been constructed for the majority of agriculturally important

species, although the density of the maps varies considerably among species. Molecular markers should not be considered as normal genes as they usually do not have any biological effect. Instead, they can be thought of as constant landmarks in the genome. They are identifiable DNA sequences, found at specific locations of the genome, and transmitted by the standard laws of inheritance from one generation to the next. They rely on a DNA assay, in contrast to morphological markers that are based on visible traits, and biochemical markers that are based on proteins produced by genes. Different kinds of molecular markers exist, such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPDs) markers, amplified fragment length polymorphisms (AFLPs), microsatellites and single nucleotide polymorphisms (SNPs).

1.3.3. Genome maps

The first reported map in livestock was for chicken in 1992, which was quickly followed by the publication of maps for cattle, pigs and sheep. Since then, the search for useful markers has continued and further species have been targeted, including goat, horse, rabbit and turkey (see www.thearkdb.org/ for the current status regarding some major livestock species). Microsatellites markers have been of major importance.

Several key publications have reported progress on the linkage map of the sheep genome based on an international mapping flock developed in New Zealand (Maddox et al., 2001). The latest sheep linkage map (version 4.3) comprises 1256 gene markers mapped to unique locations (Maddox, 2005) and most genomic regions are well covered with a maximum gap of 20 cM. However, there are quite a number of markers of low quality, so a typical genome scan would leave a number of gaps. Most of the markers are microsatellites. The total number of sheep loci listed in the ARKdb database (http://iowa. thearkdb.org) contains more than 2000 markers, but many of these are not on the linkage map. The development of the sheep genome map runs somewhat behind developments for other livestock species because of substantially lower investments. Nevertheless, at the DNA level

where the sequence can be aligned, there is a ~90 percent homology with the cattle sequence and through gene coding regions ~96 percent, and the sequencing of the cattle genome will therefore greatly enhance the development of the genome map in sheep. There is generally good agreement between sheep and cattle maps, with 598 mainly anonymous common microsatellite loci, i.e. gene markers can be linked to a comparative map. Based on sequence information in other mammals (mainly cattle) and sheep GeneBank sequences, comparative mapping can be used to construct a predicted sheep map.

The number of single nucleotide polymorphism (SNP) markers in sheep is about ~60000, but with the cattle sequence known and with an international collaborative sheep bacterial artificial chromosome (BAC)-end sequencing project under way, it is expected that there will be a large number of SNPs available for sheep. This will form a set of markers that would allow high-density genome-wide scans.

1.3.4. Quantitative Trait Loci (QTL) mapping in domestic animals

The ability to mate animals at will has allowed animal breeders to generate experimental crosses de novo for the purpose of gene mapping. Typically, F1 animals are generated by crossing breeds that are highly divergent for the traits of interest, and the F1 animals are then either intercrossed (F2) or backcrossed (BC) to one of the parental lines. Although the BC design may provide more detection and resolution power when pursuing specific QTL, in most circumstances the F2 design is preferred because of its polyvalence (18). In animal breeding, the choice between an F2 or a BC is most often dictated by practical considerations. A BC pedigree only requires the generation of a few F1 males that can then be mated to available pure-bred females. An F2, however, requires the additional generation of sufficient numbers of F1 females.

A more popular design is the “granddaughter design,” which consists of large sets of paternal half-brothers (Weller et al., 1990). The “granddaughter design ”(GDD) is based on a series of paternal half-brother pedigrees,

typically 10 to 20 sire groups with 10 to 100 sons each. In the granddaughter design, the phenotypes used for QTL mapping are the males' breeding values estimated from the performances of their respective daughters by progeny testing. Progeny tests are typically based on at least 50 daughters, The granddaughter design requires 2,5 –3 times fewer genotyped animals than does the daughter design (Georges et al. 1995, Grisart et al. 2002.).

Many quantitative trait loci (QTLs) that affect a broad range of phenotypes - including growth, body composition and fertility - have already been mapped with high confidence in the different livestock species and are awaiting further characterization (Andersson et al. 2004). However, the lack of genomic resources in domestic animals, as compared with species such as human and mouse, has hampered progress in gene mapping and identification.

Most biological traits and all common diseases in humans have a multifactorial (or complex) inheritance, which indicates that they are influenced by numerous genes and environmental factors. A chromosomal region that contains one or more genes that influence a multifactorial trait is known as a QTL (Mackay 2001, Andersson 2001) .The use of segregation analysis in informative families or experimental crosses to map QTLs is well established (Lynch 1998, Jansen 2003, Hoeschele 2003). The power of such analyses to detect and map QTLs depends on how large a fraction of the phenotypic variation is explained by a given locus and the size of the segregating population. The principal challenge with multifactorial traits lies not in detecting QTLs, but in unravelling the genes that underlie them. Despite large efforts to identify the genes that affect multifactorial traits, in particular those that are involved in common human diseases, there are few success stories (Darvas et al. 2002, Glazier et al. 2003) .The identification of genes and mutations that underlie QTLs is problematic for several reasons. First, it remains difficult to determine the exact chromosomal location of a QTL. For these reasons, QTLs are often mapped to chromosomal regions that are over 20 centiMorgan (cM) long (~20 megabase pairs (Mb)) and that might contain several hundred genes. Second, most QTLs have a mild

phenotypic effect, so the mutations that cause them are difficult to distinguish from neutral polymorphisms.

Another factor that complicates the identification of QTL mutations is that it is likely that a good proportion of these are regulatory mutations. The ability to spot and evaluate functionally important mutations in non-coding regions is still poorly developed.

There are still relatively few examples for which the mutations that underlie mapped QTLs have been identified in domestic animals. These few examples have been identified either because a gene that causes a monogenic trait has pleiotropic effects on several complex traits, or by adopting a positional candidate approach combined with linkage-disequilibrium analysis.

On the basis of the relationship between the observable polymorphic loci and the traits of interest, three types of markers can be considered (Dekkers 2004):

1) LE markers: loci that are in population wide linkage equilibrium with the functional mutation in outbred populations;

2) LD markers: loci that are in population- wide linkage disequilibrium with the functional mutation;

3) direct markers: loci that code for the functional mutation;

The LE markers can be readily detected on a genome-wide basis by using breed crosses or analysis of large half-sib families within the breed. Such genome scans require only sparse marker maps (15 to 50 cM spacing, depending on marker informativeness and genotyping costs; Darvasi et al., 1993) to detect most QTL of moderate to large effects. Many examples of successful applications of this methodology for detection of QTL regions are available in the literature.

The LD markers are by necessity close to the functional mutation for sufficient population-wide LD between the marker and QTL to exist (within 1 to 5 cM, depending the extent of LD, which depends on population structure and history). The LD markers can be identified using candidate genes or fine-mapping approaches.

In the positional candidate strategy linkage analysis is used to map the locus to a specific chromosomal region. This region is subsequently scrutinized for candidate genes that might influence the trait being studied. The next step is then to search for causative mutations in the candidate gene. This approach is the most common strategy for dissecting monogenic traits in mammals as there are complete genome sequences for several species and the functional characterization of genes is continuously improving. However, the generally poor resolution of initial QTL mapping means that this approach is more difficult to apply to multifactorial traits. Specifically, the region that a QTL is mapped to might contain too many plausible candidate genes and even several poorly characterized genes that cannot be excluded as candidates. Occasionally, however, positional candidate cloning can be a quick way of identifying a causative gene that can be confirmed by further genetic data or functional assays.

This markers (i.e., polymorphisms that code for the functional mutations) are the most difficult to detect because causality is difficult to prove and, as a result, a limited number of examples are available.

The three types of marker loci differ not only in methods of detection, but also in their application in selection programs. Whereas direct markers and, to a lesser degree, LD markers, allow for selection on genotype across the population because of the consistent association between genotype and phenotype, use of LE markers must allow for different linkage phases between markers and QTL from family to family. Thus, the ease and ability to use markers in selection is opposite to their ease of detection and increases from direct markers to LD markers and LE markers.

1.3.5. Quantitative Trait Nucleotide (QTN) identification in domestic animals

Currently, four causal QTNs with strong support have been identified by positional cloning in domestic animals: the diacylglycerol acyltransferase 1 (DGAT1 K232A) (Grisart et al. 2002, Grisart et al 2004, Winter et al. 2002) and ABCG2 Y581S (ATP-binding cassette superfamily G member 2

Transporter; Cohen-Zinder et al. 2005) mutations influencing milk composition in cattle, and the IGF2 (insulin-like growth factor 1) intron 3-3072(G-A) (Van Laere et al. 2003) and MSTN (myostatin) 3'untranslated region (UTR) g+6723(G-A) (Clop et al. 2006) mutations influencing muscle mass in pigs and sheep, respectively. A small number of additional putative QTNs have been reported but require additional scrutiny in independent samples (Schnabel et al. 2005, Sugimoto et al. 2006). This number may not seem like much, but it compares favorably with results achieved in other organisms, including man and mice, which receive considerably more attention and support (Glazier et al. 2002). What is most striking in domestic animal studies is the ability to identify regulatory mutations. It is much more difficult to predict the effect a putative regulatory mutation has on gene function than the effect of a mutation that alters the primary protein sequence. To the IGF2 intron 3-3072(G-A) and MSTN 3'UTR g+6723(G-A) regulatory mutations, one may add the CLPG mutation, although the corresponding callipyge trait was approached more as a binary than as a quantitative trait (Freking et al.2002, Shuster et al. 1992). The intron 3-3072(G-A) mutation in the imprinted porcine IGF2 gene invalidates a muscle-specific silencer located in intron 3 operating after birth. The mutation abrogates binding of an as-of-yet unidentified transacting factor. As a result, animals inheriting the intron 3-3072(G-A) mutation from their father have threefold higher levels of IGF2 mRNA in muscle, which leads to increased muscle mass and decreased fat deposition (Van Laere et al. 2003). The Texel MSTN g+6723(G-A) mutation creates an illegitimate target site for microRNAs miR-1 and miR-206 in the gene's 3'UTR. As a result, mutant MSTN mRNAs are translationally repressed, causing decreased levels of the MSTN protein and increased muscle mass. The g+6723(G-A) mutation is particularly interesting as it is the prototype of a new class of mutations susceptible to generate both hypo- [gain of a microRNA (miRNA) target site] and hypermorphic (loss of a miRNA target site) alleles that might make significant contributions to the heritability of a broad range of complex traits (Clop et al. 2006, Georges et al. 2006). The CLPG mutation also inactivates

a muscle-specific silencer that controls the expression of the DLK1-GTL2 cluster of imprinted genes after birth. The CLPG mutation is located in the middle of the 90-Kb intergenic region separating DLK1 and GTL2. Heterozygous animals receiving the CLPG mutation from their father exhibit ectopic expression of the paternally expressed DLK1 mRNA and DLK1 protein in muscle, which causes the callipyge muscular hypertrophy (Charlier et al. 2001, Davis et al. 2004). Intriguingly, homozygous CLPG/CLPG animals show increased levels of DLK1 mRNA, but not of DLK1 protein in skeletal muscle and hence do not express the callipyge phenotype. There is increasing evidence that this is due to translational repression of the padumnal DLK1 transcripts by madumnal miRNAs (Davi set al. 2005). It is remarkable that both the Texel and callipyge muscular hypertrophies involve miRNA-mediated mechanisms, highlighting the importance of this newly discovered mode of gene regulation (Georges et al. 2007).

1.3.6. Mapping QTL in dairy sheep

In the European Union, a series of regional projects started at the end of the 1990s to analyse the genetic variability of economically relevant traits. Most investigations focused on production traits due to the lack of phenotypic information on functional traits. Some of these projects were federated in the EC-funded Genesheepsafety project to investigate both production and functional traits.

The countries involved in this project were United Kingdom, France, Spain and Italy. In each country an experimental population was created: Scottish Blackface population in UK; French commercial dairy sheep population (Lacaune, Basco-Bearnaise and Manech) in France; Churra population in Spain and Sardinian x Lacaune backcross population in Italy. Latter population represents the basis of this study.

2. Objectives

The main aims of this research were:

- ✓ To improve the significance level and refine the location of one QTL found for Conjugated Linoleic Acid (CLA) and Vaccenic Acid (VA) ratio on ovine chromosome 22 in the experimental Sardinian x Lacaune backcross population;
- ✓ To validate the found QTL in Sardinian pure breed by investigation of sire families of the 7/8 Sardinian x Lacaune experimental population;
- ✓ To identify a positional candidate gene;
- ✓ To find causal mutations along this gene associated with the CLA and VA ratio variability in sheep milk.

3. Materials and methods

3.1. General experimental approach

Due to the evolution of the EU agricultural policy and consumers demand, more attention has been now given to traits related to the reduction of production costs (milkability, functional traits, longevity), health (resistance to mastitis or parasitic diseases) and safety food (milk content in fatty acids related to human health). In most situations, only a part of these new traits is extensively recorded in the nucleus flocks of the purebred breeding schemes (Barillet, 1997; Rupp et al., 2002, Sanna et al., 2002). Therefore research combining classical quantitative approach and QTL detection is needed, either on-farm by implementing experimental recording schemes, or in experimental flock especially for traits difficult and costly to record.

To find marker haplotypes or gene polymorphisms, that could be used for marker assisted or gene assisted selection, AGRIS Sardinia planned a complex research activity. The main steps of this approach are:

- Genome scan in a back-cross Sardinian X Lacaune experimental population in order to identify putative QTL regions;
- Densification of some regions of interest to confirm QTL by new QTL detection analysis and to perform a positional candidate gene approach;
- Validation of identified marker haplotypes or candidate gene mutations in the pure breed population;
- Sequencing of positional candidate gene and/or closely linked haplotype markers;
- Application of Marked Assisted Selection.

In a first step a Sarda x Lacaune backcross resource population was procreated in 1999 by INRA (France) and AGRIS (Italy) in order to detect QTL both on milk production and traits difficult to measure by means of whole genome intermediate density genetic map. Crosses between breeds to produce F2 or backcross experimental population allow to exploit linkage

disequilibrium for genes differing between breeds and to detect genome regions controlling the traits of interest. This experiment is expected to detect genome regions associated with traits of interest even if with low precision of location. Therefore the global approach forecasts the densification of these genome regions by the addiction of further markers. In fact in the backcross experiment the average distance between markers is approximately 20 cM leading to high confidence interval of the QTL location. Densification should reduce the average distance between markers to 3-5 cM, in order to permit a more precise location of the found QTL. Once the QTL has been confirmed with denser map in the backcross population, surrounding markers will be used to verify the segregation of the QTL in pure breed population. With this aim a further experimental population has been created by mating Sardinian rams with the backcross ewes (see following paragraphs for details on this population). Also this population is organized in half-sibs families so following a classical daughter design. Since this experimental design implies the contrast of chromosomes segments inherited from the sire, it should permit to verify QTL segregation in the Sardinian pure population.

In a second phase, functional candidate genes among those located in the found QTL region, will be identified to be sequenced. This approach should permit to detect causative mutations responsible of the traits variability.

3.2. Experimental populations

3.2.1. The Backcross population experiment

This study is based on a experimental population Backcross Sardinian x Lacaune. The Sarda and the Lacaune are the two most numerous French and Italian dairy sheep breeds which differ for a lot of traits as body size, growth rate, wool, prolificacy, milk yield level and milkability and for their dairy selective history (Barillet et al., 2001; Carta et al. 2002).

In 1997, 14 elite Lacaune rams from France were mated in Italy by artificial insemination to 184 Sardinian ewes to produce 77 F1 males. Among these, 45 were raised in the experimental farm of “Bonassai” in the North of

Sardinian. Ten of these, deriving from different Lacaune sires were chosen, on the basis of their development growth and their mating ability, as sires of the experimental backcross population. In 1998, these F1 rams were mated, by artificial insemination and natural mating to 2,719 Sardinian ewes. Nine backcross families derived from ewes belonging to AGRIS while one (family 10) derived from ewes belonging to a private farmer. Finally 968 backcross females, deriving from 10 F1 Lacaune x Sardinian rams and 835 Sardinian ewes, born between 23 December 1998 and 19 January 1999 were bred (Table 3-1).

BC Sires	Sardinian dams	Daughters	Ewes with lactation recorded			
			2000	2001	2002	2003
1	92	109	96	96	96	81
2	84	94	91	86	84	74
3	105	121	112	113	100	82
4	101	114	101	103	86	70
5	75	83	76	75	74	58
6	92	112	102	100	88	78
7	71	84	78	77	75	69
8	74	87	80	84	78	71
9	78	88	83	79	72	59
10	63	76	68	72	66	63
Total	**835**	**968**	**887**	**885**	**819**	**705**

Table 3-1 Structure of the backcross Sardinian x Lacaune population.

This design corresponded to a classical daughter design for QTL detection (Weller et al 1990). Average family size was 96.8, ranging from 76 to 121, ewes per family (Table 3.1). From August 1999 until their slaughter in 2004, the ewes were bred in an experimental farm of AGRIS (Monastir). The farm (160 ha -1/3 of which irrigated plan) is located in the South of Sardinia with a dry Mediterranean climate (average rainfall 400 mm/year concentrated between November and March and high temperatures during summer, up to 40° C). The ewes were fed with the same regimen based on 4-5 hours of grazing irrigated mixed swards of ryegrass and berseem clover with important supplementations of lucerne hay, maize silage and concentrates, particularly in winter and late spring. The first mating was in September 1999 while the 4 following mating seasons occurred in June 2000-2001-2002-

2003. Thus lambings occurred in winter 2000 (first lactation) and autumn 2000, 2001, 2002, 2003. The ewes were bred in 3-4 groups depending on the lambing period. After 3 (1st and 2nd lactation) or 4 weeks (3rd and 4th lactation) of suckling they were milked twice a day by machine at 5.00 a.m. and 3.30 p.m., in order to limit differences in inter-milking interval. In July they were progressively dried off and only milked at morning.

Many phenotypic measurements have been taken from the backcross Sardinian x Lacaune experimental population (Table 3-2). Most of these measurements were performed using specific softwares on hand-held computers and electronic devices for electronic identification, weighting and milk recording. These tools enable on-farm verifications and corrections if needed.

Traits	**Periodicity/Tools**
Production traits:	
Daily milk yield	Three times per month/INRA electronic jar
Protein and fat content	Bi-monthly/INRA electronic jar
Growth (as ewe lamb)	Bi-monthly body weight/electronic balance
Body Weight (adult ewe)	Monthly body weight/electronic balance
Body condition score (adult ewe)	Monthly subjective score
Wool weight	Once a year/electronic balance
Wool quality	Ewe lamb/objective measurements & score
Milkability and udder morphology	
Kinetics of milk flow	Three times per month/INRA electronic jar
Udder morphology type traits	Monthly in L1, 3 times/year L2-L3-L4 /subjective score
Udder measurements	Once a year in L1 & L2/numeric picture analysis
Health traits	
Udder health : somatic cell count	Bi-monthly/INRA electronic jar
Udder health : clinical mastitis detection	daily/visual detection +microbiological assay
Nematode parasite resistance	2- 3 times per year /faecal eggs count
Oestrus ovis resistance	Once a year/antigen ELISA test
Reproductive traits	
Ovulation rate	Once a year/laparoscopy
Fertility (after synchronization)	Date of lambing on the controlled cycle
Prolificacy	Litter size at lambing
Ability of out-of season mating	Once a year before mating/Progesterone assay
Anti PMSG antibody	3 ELISA tests around AI
Embryo mortality	Radio-immuno assay of PSPB
Milk quality traits	
Milk fatty acids composition	Once a year /gas-chromatography

Table 3-2 Description of the main phenotypic measurements performed on the backcross Sardinian x Lacaune population.

A genome- scan approach was performed on this population using a panel of 155 microsatellites made-up by INRA. The markers were chosen to have an average distance between them of 20 cM, easy of scoring and number of alleles. At the end of the project, due to the quality of some commercial primers as well the low information content of some of the tested microsetallites, 145 markers were available (Table 3-3) respect to the 155 needed.

OAR	Markers	n. Marker
1	BMS2833; BMS0835; ILSTS044; BMS862; ILSTS029; MCM058;BL41;BMS0963;BMS0574;BMS2572;INRA49;LSCV06; MAF109; LSCV105; BMS1789; BMS2263	16
2	CSSM47; MCM064; BMS2072; ;BMS1341; TGLA010; BMS1591; OAECP79; ILSTS030; LSCV22; BMS0778; BM6444; BMS0356;LSCV38; OARFCB11;	14
3	ILSTS045 ; BMS0460; OARFCB129; INRA131; RM096; BMS2569 ; BMC1009; OARCP43; RM154; BMS0772	10
4	BMS1788; MCM218; BMS1237; MAF50; OARHH35; OARHH64	6
5	RM006; BMS2258; MCM527	3
6	BM9058; INRA133; ILSTS090; OARAE101; BMS0360; OARJMP08; BL1038	7
7	BMS0528; BMS0861; BMS0419; BMS2641; BP31; ILSTS005	6
8	BM2504; BMS0434; BMS1290; BMS1724; EPCDV016; BMS1967	6
9	CSSM66; ILSTS011; LSCV32; BM302; OARCP9	5
10	BMS2252; BMS0712; ILSTS056; BMS1316	4
11	HEL10; IDVGA46; BM17132; MAP2C MCM120	5
12	HUJ614; BMS0109; BMS1185; IDVGA69; HUJ625	5
13	BMC1222; ILSTS059; BMS1352; PRP; BMS1669;OARAE016	6
14	TGLA357; BMS2213; LSCV29; BM7109; INRA210;	5
15	MAF65; BMS0812	2
16	BM1225; MAF214; LSCV08; MCM150	4
17	OARVH98; OARVH116; OARFCB48; TGLA322	4
18	BM3413; BMS2815; BM7243; OARHH47; TGLA122; BMS1561	6
19	BMS0517; OARAE119; BM3628; LSCV14	4
20	INRA132; BM1258; OLADRBP; OARHH56	4
21	ILSTS019 ; OARVH110; OCAM; MCM135; BMS1948	5
22	BMS0651; BM4505; BMS882; MCM373	4

OAR	Markers	n. Marker
23	BMS2526; BMS0066; MAF35; URB031;	4
24	ILSTS102; BM4005; OAREL001	3
25	OARVH41; BMS1714	2
26	BMS2104; LSCV41; CSSM43; OARJMP58; BM0203	5

Table 3-3 Panel of markers available for the genome scan of the backcross Sardinian x Lacaune population.

QTL were detected on different analysed traits. Particularly QTL were detected for somatic cell count (Rupp et al.,2003), nematode parasite resistance (Scala et al. 2002; Moreno et al., 2006), udder morphology (Casu, 2004a; Casu et al., 2004b), kinetics of milk emission (Casu et al., 2004c), wool production (Allain et al 2006), CLA in milk (Carta et al., 2003a; Carta et al., 2006) and other fatty acid contents (Carta et al., 2008).

3.2.2. 7/8 Sardinian x Lacaune population

7/8 Sardinian x Lacaune population was procreated, between 2001 and 2003, by mating the backcross (BC) ewes with 18 Sardinian rams in the farm of “Monastir”. Sires were chosen among the proved rams of the artificial insemination centre as representatives of the Sardinian breed. They belonged to 12 flocks from different regions of Sardinian. Table 3-4 shows the impact of these sires on the Sardinian flock-book population.

Seven Sardinian sires were mated by artificial insemination in the mating season 2001 (2nd lactation of BC population). In the mating seasons 2002 and 2003 six and seven Sardinian sires respectively were mated by natural mating. Sires 7 and 12 were used for two mating seasons, 2001-2002 and 2002-2003 respectively. The other sires were mated only for one year.

The 7/8 Sardinian x Lacaune population was completed in autumn 2003. It was composed of 800 ewes organized in 18 half-sib families with 44.4 (ranging from 32 to 95) daughters per sire on average (Table 3-5). The availability of both daughters and dams genotypes increases the power of the experimental design, making up for smaller family size respect to the BC population.

475 backcross dams procreated this population. Out of these, 146 had daughters in two families and 26 in three families, obviously in different years. Moreover, seven 7/8 ewes were daughters of not genotyped BC ewes. This population was raised in the farm of "Monastir" by the same management described for BC population. The first mating was at the age of around 10 months, in the autumn following the birth. The first lambing was in the following winter whereas successive lambings always occurred in autumn. After 4 weeks of suckling, ewes were milked twice a day by machine. In July they were progressively dried off and only milked at morning. The first cohort of ewes (born in 2001) was simultaneously slaughtered on June 27th, 2006 at the end of their 4th lactation (Table 3-6).

7/8 Sires	District	Sardinian daughters	Sardinian flocks	Year of 1st EBV	Seasons performed
1	SS	61	33	2002	3
2	SS	80	28	2002	2
3	SS	277	109	2000	6
4	NU	303	133	1998	9
5	NU	99	53	2001	5
6	SS	358	150	1999	8
7	NU	224	101	1999	6
8	CA	103	46	2000	4
9	CA	70	30	2001	4
10	SS	61	33	2001	3
11	NU	138	62	1999	8
12	CA	117	54	1999	4
13	SS	210	89	2000	6
14	SS	100	55	2002	3
15	NU	55	24	2003	1
16	CA	138	73	2001	6
17	NU	31	20	2003	1
18	SS	49	22	2002	1

Table 3-4 Impact of the 7/8 Sardinian x Lacaune sires in the Sardinian selection scheme.

7/8 Sires	Dams (*)	Year of birth			Total
		2001	2002	2003	
1	27	32			32
2	30	37			37
3	36	39			39
4	35	42			42
5	39	48			48
6	44	52			52
7	79	47	48		95
8	28		33		33
9	26		34		34
10	36		47		47
11	37		51		51
12	44		34	12	46
13	21			28	28
14	29			33	33
15	37			43	43
16	39			44	44
17	38			44	44
18	47			52	52
Total	**672**	**297**	**247**	**256**	**800**

(*) some dams lambed in different years and in different families

Table 3-5 Structure of the 7/8 Sardinian x Lacaune population.

7/8 Sires	Daughters	Ewes with lactation record				
		2003	2004	2005	2006	2007
1	32	29	29	24	24	Culled
2	37	29	30	26	25	Culled
3	39	34	35	31	26	Culled
4	42	37	39	31	28	Culled
5	48	46	44	30	29	Culled
6	52	45	38	29	30	Culled
7	95	41	86	82	74	1/2Culled
8	33		26	32	30	4th lat
9	34		30	31	31	4th lat
10	47		43	42	42	4th lat
11	51		43	48	47	4th lat
12	46		26	40	38	4th lat
13	28			24	25	3rd lat
14	33			33	29	3rd lat
15	43			39	36	3rd lat
16	44			43	38	3rd lat
17	44			42	43	3rd lat
18	52			48	48	3rd lat
Total	**800**	**261**	**469**	**675**	**643**	

Table 3-6 Number of 7/8 ewes with lactation record.

Figure 3-1 shows the complete structure of backcross and 7/8 Sardinian x Lacaune populations.

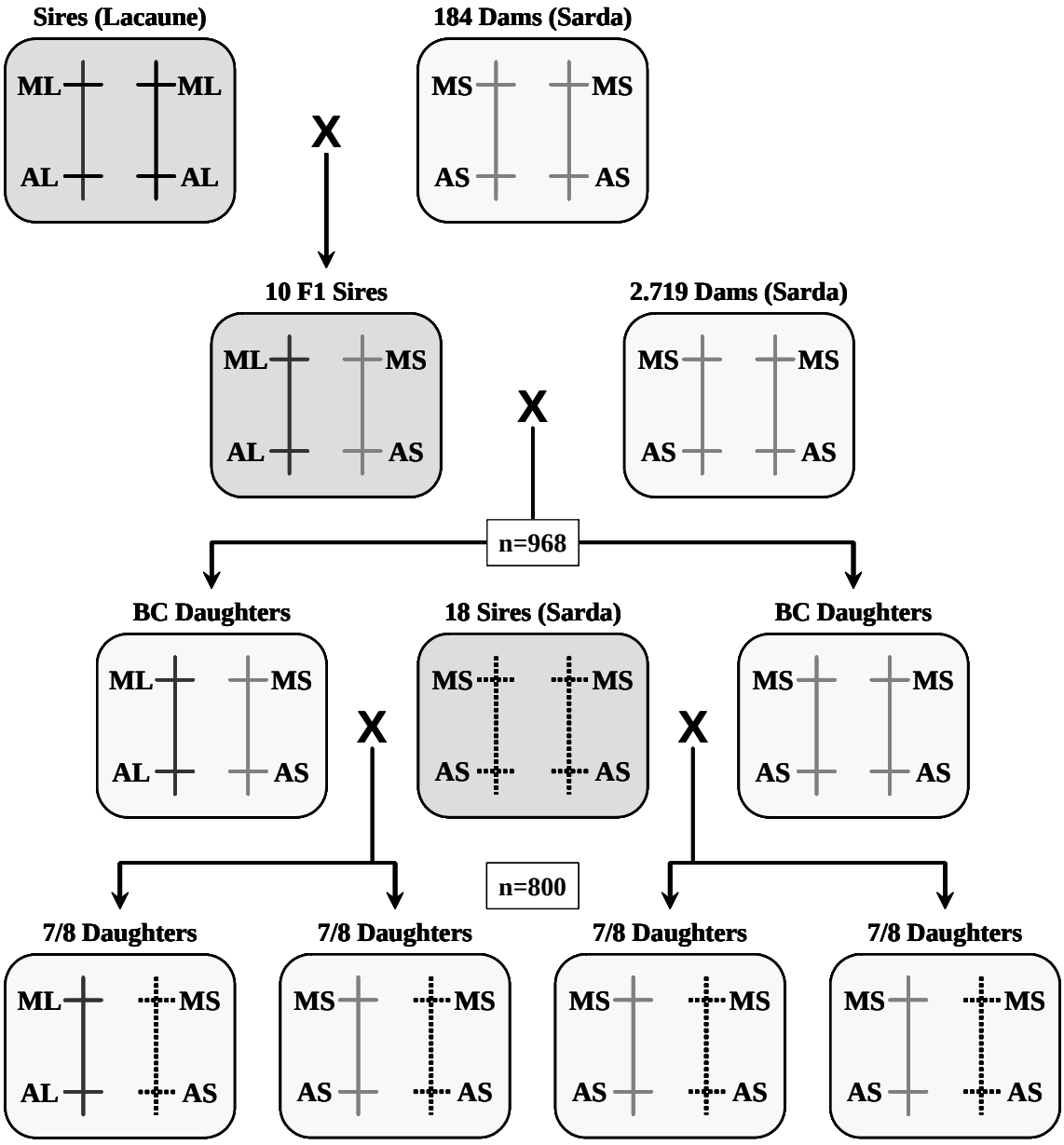

Figure 3-1 Structure of Backcross and 7/8 Sardinian x Lacaune populations. ML: Lacaune Marker Allele; AL: Lacaune QTL allele; MS: Sardinian Marker Allele; AS: Sardinian QTL allele.

3.3. Fatty acid content analyses

Milk yield was recorded by automatic recording device developed by INRA (European brevet N°94916284.6. Guillonet et al., 1990). Fat and protein contents were determined by infrared method by Milkoscan (Foss Eletric, Hillerod, Denmark).

FA content was analysed as follows:

- ✓ fat separation by centrifuging at low temperature 4°C;
- ✓ storage of individual cream at −20 °C;
- ✓ oil separation by thermal shock and centrifuging;
- ✓ acid trans–methylation.

Fatty acid methyl esters (FAME) were determined by gas chromatography using a VARIAN GC 3600 equipped with FID and a fused silica capillary column (SP 2560 Supelco),100 m ×0.25 mm i.d., film thickness 0.20µm. Helium was used as the carrier gas at a flow of 1 ml/min. The split ratio was 1:100. The oven temperature was programmed at 75 ˚C and held for 1.50 min, then increased to 190 °C at a rate of 8 °C/min, held for 25 min, increased to 230 °C at 15 °C /min, held for 4.47 min.The temperatures of the injector and of the detector were set at 290 °C. Fatty acids were identifyed confronted with standards (C5; C9;C13;C19) retention time.

3.4. Molecular tools

3.4.1. Genomic DNA extraction

Two methods of DNA extraction were used:

- ✓ a rapid technique for the microsatellites analysis;
- ✓ a commercial kit for sequencing analysis.

3.4.1.1. Rapid DNA Extraction

Blood samples were collected in 5 ml vials vacutainer containing the anticoagulant EDTA and DNA was extracted from frozen white blood cells using a rapid technique with the ultimate intent of obtaining a final concentration of 10 ng/µl following these steps:

- ✓ to remove red cells, blood samples were first submitted to several washings with NE (NaCl 10 mM, EDTA 10 mM);
- ✓ NaOH 200 mM was added and samples were incubate at 65°C for 1h to induce white cells lysis;
- ✓ a TrisHcl 100mM/HCl 100 mM mix 1:1 was added to the samples to obtain a final pH= 7.

3.4.1.2. Commercial KIT extraction

- ✓ FlexiGene DNA Kit (Qiagen) following the supplier's protocol with some modifications was used for blood samples;
- ✓ Puregene DNA Kit (Gentra) following the supplier's protocol with some modifications was used for semen samples.

3.4.2. The Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a revolutionary method developed by Kary Mullis in the 1980s. PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. (Saiki et al., 1985; Mullis, 1990).

The enzyme is proofreading and has 5'-3' exonuclease activities to correct for misincorporated bases. However, errors can occur, with frequencies varying between 10^{-9} and 10^{-10} per base replicated (Echols and Goodman, 1991), depending on the size of fragment being amplified and reaction conditions (concentration of magnesium chloride and dNTPs, pH, temperature).

DNA polymerase enzyme synthesize a complementary strand of a targeted segment of DNA. The reaction needs the DNA polymerase enzyme and the DNA segment to amplify, a large quantity of the four nucleotides (A=Adenine, T=Thyimine, G=Guanine, C=Cyitosine) and large quantities of two oligonucleotides DNA fragments called primers, complementary to the first part of the segment of DNA that is being copied (they attaches to the beginning of the template strand by base pairing).

Currently, *Thermus aquaticus* (Taq) DNA polymerase is widely used for DNA amplification.

The three main steps in a PCR reaction are repeated for 30 to 40 cycles. This can be done on an automated thermalcycler, which can heat and cool the tubes with the reaction mixture in a very short time. The steps are:

1. DNA denaturation at 94-95°C. Heating melts the double stranded DNA in 2 single strands, all enzymatic reactions stop (for example : the extension from a previous cycle);
2. primers annealing at 54-62°C (depending on the primer sequence). The primers are jiggling around, caused by the Brownian motion. Ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer, that it does not break anymore;
3. extension at 72°C. This is the ideal working temperature for the polymerase. The primers, where there are a few bases built in, already have a stronger ionic attraction to the template than the forces breaking these attractions. Primers that are on positions with no exact match, get loose again (because of the higher temperature) and don't give an extension of the fragment. The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template).(Figure 3.2).

The three described steps of polymerase chain reaction take less than two minutes and the whole process can be carried out in the same vial. At the end of a cycle, the DNA fragment in the vial is duplicated. Therefore, after 30 cycles, aproximately a million copies of a sequence of DNA can be obtained. About 1 million copies can be made approximatively in three hours. (Figure 3.3).

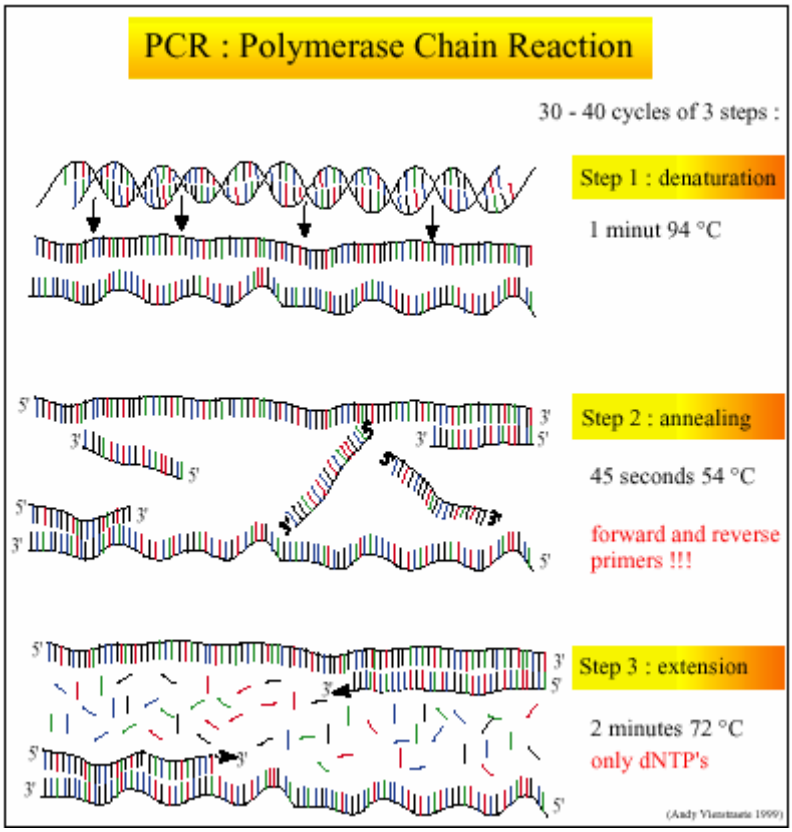

Figure 3.2 Scheme of the three PCR steps.

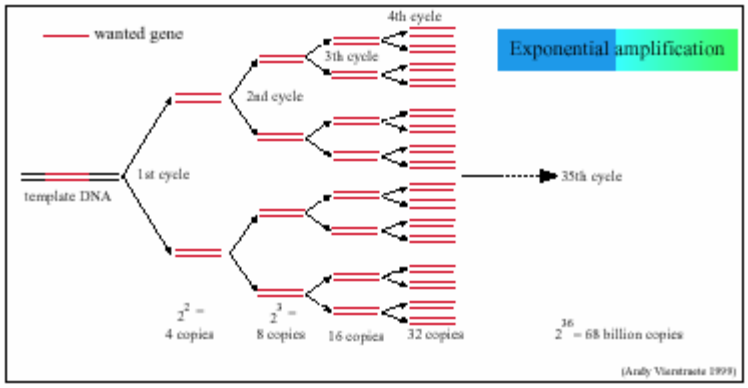

Figure 3.3 PCR: exponential amplification.

3.4.3. Microsatellite loci

Microsatellites are stretches of DNA, generally in the genome non-coding regions, that consist of tandem repeats of a simple sequence of nucleotides. The repeated units are generally di-nucleotides, although tri- tetra- or even penta-nucleotides exist (Figure 3.4)

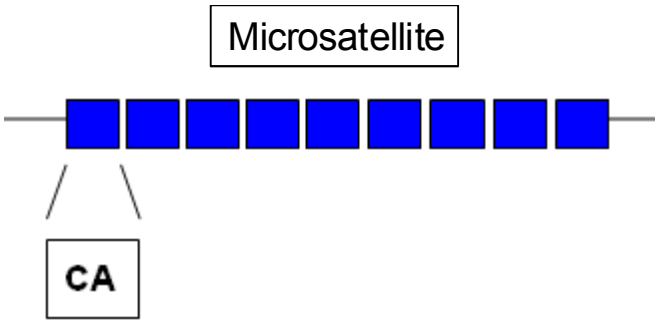

Figure 3.4 Microsatellite structure: in the figure each block is a di-nucleotide repetitions.

Microsatellite alleles differ in the number of repetitions, for example, one allele may be characterized by 11 repetitions of a CA motif, and another allele by 13 (Figure 3.5).

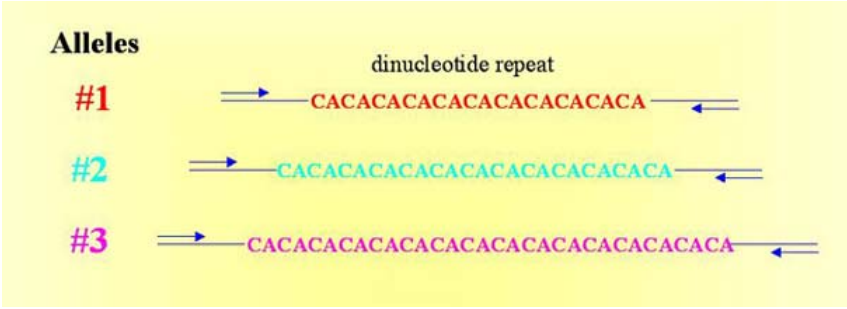

Figure 3.5 Alleles of microsatellites differ in the number of repetitions.

In a population, many alleles at a single locus may exist, with each allele having a different length. A homozygous individual in a locus is characterized by the same number of repetitions on both chromosomes, whereas a heterozygous individual has different numbers of repetitions. Using the polymerase chain reaction (PCR) and an electrophoretic run, the number of repetitions can be easily detected. Microsatellites are very useful because:

- ✓ they are codominant: heterozygous can be distinguished from homozygous;
- ✓ they are PCR-based, so we need only tiny amounts of tissue;
- ✓ they are highly polymorphic.

The abundance and high level of allelic variations at microsatellite loci in the genome of many organisms has made them popular genetic markers within a wide range of analyses (McDonald et al.,1997), also because they may be transferred from one species to another. They are very important tool in:

- ✓ studies of QTL detection (Heyen *et al.*, 1999; Schrooten *et al.*, 2000; Malek *et al.*, 2001; Nezer *et al.*, 2002; Carta *et al.*, 2003; Barillet *et al.*, 2005);
- ✓ Marker Assisted Selection (Dekkers *et al.*, 2002; Dekkers, 2004);
- ✓ genome mapping;
- ✓ biomedical diagnosis;
- ✓ biological/evolutionary as markers for parentage analyses;
- ✓ studies on the genetic structure of subpopulations and populations (Michalakis *et al.*, 1996);
- ✓ phylogeographic studies that try to explain the concordant biogeographic and genetic histories of the floras and faunas of large-scale regions;
- ✓ biodiversity studies, particularly on big scale (ECONOGENE http://www.econogene.eu/;; Canòn *et al.*, 2006; European Cattle Genetic Diversity Consortium, 2006; Pariset *et al.*, 2006a; Pariset *et al.*, 2006b; Sechi et al 2007).

3.4.4. Sequencing

Currently, direct sequencing is one of the high throughput methods for mutation detection, and is the most accurate method to determine the exact nature of a polymorphism.

The purpose of sequencing is to determine the order of the nucleotides of a DNA segment, previously amplified. PCR or cloning, using a mix of dNTP's and ddNTP's (chain terminators). These dideoxynucleotides are the chain-terminating nucleotides, lacking a 3'-OH group required for the formation of a phosphodiester bond between two nucleotides during DNA strand elongation. Three main steps are repeated for 30 to 40 cycles likes in PCR:

1. denaturation: heating to 95°C melts the double stranded DNA in 2 single strands;
2. annealing: at 50-62°C (depending on the primer sequence). In sequencing reactions the template is only one DNA strand;
3. extension: at 60°C. Normally the working temperature for the polymerase is 72 °C but, because it has to incorporate ddNTP's which are chemically modified with a fluorescent label, the temperature is lowered so it has time to incorporate the 'strange' molecules. The primers, where there are a few bases built in, already have a stronger ionic attraction to the template than the forces breaking these attractions. Primers that are on positions with no exact match, come loose again and don't give an extension of the fragment. The bases (complementary to the template) are coupled to the primer on the 3'side (adding dNTP's or ddNTP's from 5' to 3', reading from the template from 3' to 5' side, bases are added complementary to the template). Incorporation of a dideoxynucleotide into the nascent (elongating) DNA strand terminates DNA strand extension because lacking the 3’-OH group, resulting in various DNA fragments of varying length. The dideoxynucleotides are added at lower concentration than the standard deoxynucleotides to allow strand elongation sufficient for sequence analysis. Because the ddNTP's are fluorescently labeled, it is possible to detect the color of the last base of this fragment on an automated sequencer (Figure 3.6).

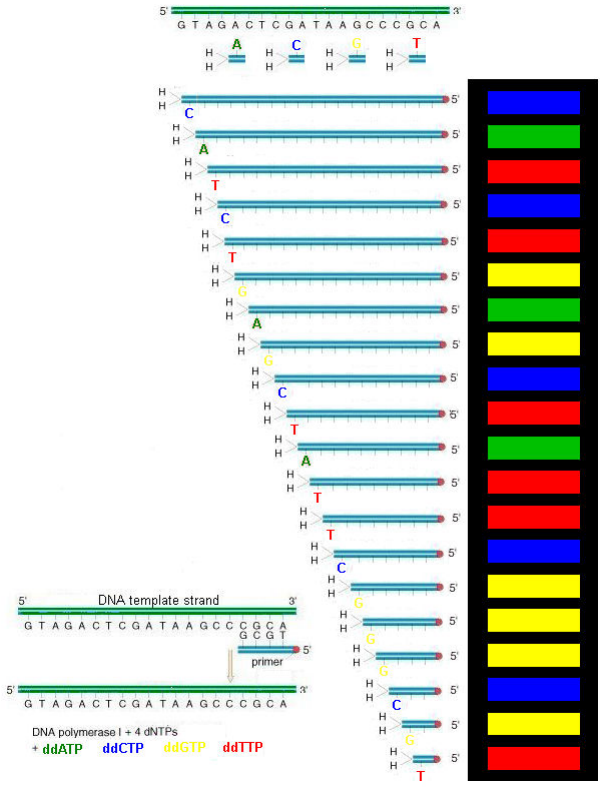

Figure 3.6 Incorporation of a dideoxynucleotide into the nascent DNA strand terminates DNA strand extension resulting in various DNA fragments of varying length.

Because only one primer is used, only one strand is copied during sequencing, there is a linear increase of the number of copies of one strand of the gene. Therefore, there has to be a large amount of copies of the gene in the starting mixture for sequencing. Suppose there are 1000 copies of the wanted gene before the cycling starts, after one cycle, there will be 2000 copies : the 1000 original templates and 1000 complementary strands with each one fluorescent label on the last base, after two cycles, there will be 2000 complementary strands, three cycles will result in 3000 complementary strands and so on (Figure 3.7).

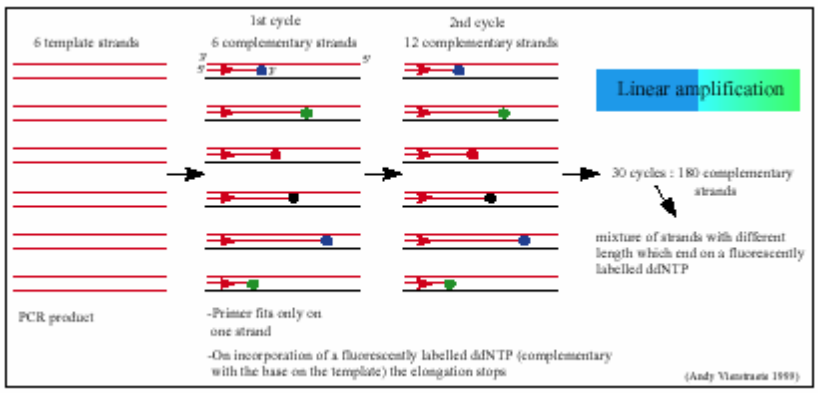

Figure 3.7 Sequencing scheme: only one strand is copied during sequencing reaction then there is a linear increase of the number of copies of one strand of the gene.

3.4.5. Moleculat Data analyses

Genotypings and sequencing were carried out using an ABI PRISM® 3100-Avant Genetic Analyzer (Applera, Foster City, CA). The genotypes were analyzed with the GeneScan version 3.1 and Genotyper version 3.0 softwares (Applied Biosystems, USA). Sequenches were analyzed with the DNA Sequencing Analysis software version 3.7 (Applied Biosystems, USA), the free software BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), the free software MutationSurveyor and the Sequencher software version 4.8 (Gene Codes Corporation, USA).

3.4.5.1. ABI PRISM® 3100- Avant Genetic Analyzer

Automated sequencing systems use fluorescent dye labelling, in combination with laser scanning and computerized data acquisition and processing to carry out the electrophoresis of up to 192 samples on a single analyses run. For fragment analysis, PCR products are tagged with fluorophores and the alleles are detected fluorescently by an argon ion laser. Reactions can be multiplexed, differentiating products by size and up to the four fluorophores.

Fluorescent labelling of DNA fragments may be performed in several ways. The most common method is to incorporate a fluorescent dye on the 5'-end of a PCR primer so that during PCR amplification either the forward or the reverse strand of DNA will be labelled. Up to four different fluorescent dyes may be used for labelling, allowing for a greater amount of multiplexing in a

single lane for higher throughput. An internal size standard, labelled with a fifth dye, provides accurate and reproducible size determination and quantification, and eliminates problems associated with lane-to-lane or run-to-run electrophoretic variability.

3.4.5.2. Softwares

Concerning microsatellites analyses, data were collected using the ABI PRISM Data collection software and are analyzed with the GeneScan 3.1 and Genotyper 3.0 softwares (Applied Biosystems, USA). Electropherograms show the migrated positions of the DNA fragments as peaks. The analysis of the results is a two-step process. The first step follows electrophoresis and includes the calculation of all peaks identified using the Genescan software (**Figure 3.8**)

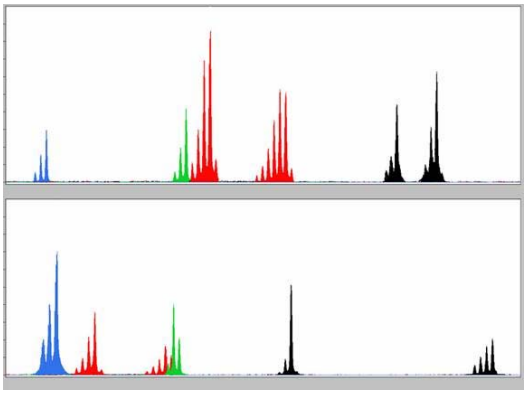

Figure 3.8 Genescan output: Multiplexed microsatellites analysis on ABI 3100. The samples are labeled with FAM (blue), VIC (green), PET (red), and NED (yellow) fluorescent dyes.

Then the raw data from Genescan are downloaded into the Genotyper software which allows the analysis of multiple samples and the identification of the only desired peaks representing the alleles (Figure 3.9).

The data are shown either as a collection of electropherograms and also in tabular form.

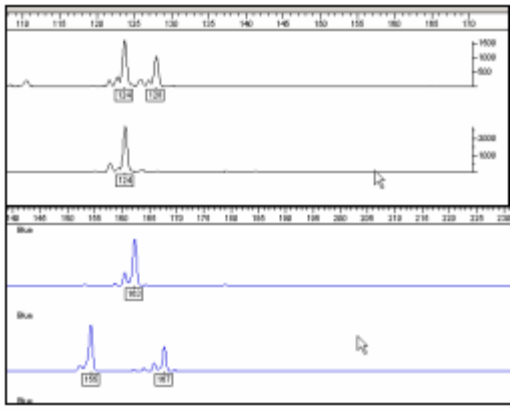

Figure 3.9 Genotyper output: 2 microsatellites labelled with different dye for 2 samples, differences between homozygous and heterozygous and the exact size of each allele are visible.

For sequencing analyses data were collected using the ABI PRISM Data collection software and analyzed with DNA Sequencing Analysis software version 3.7 (Applied Biosystems, USA) and Sequencher software version 4.8 (Gene Codes Corporation, USA). Sequencing products are tagged with fluorophores and the DNA bases are detected fluorescently by an argon ion laser. The data are shown either as a collection of electropherograms and also in tabular form.

Electropherograms show the migrated positions of the DNA bases as peaks (Figure 3.10). The analysis of the results is a two-step process. The first step follows electrophoresis uses the Sequencing Analysis software version 3.7 (Applied Biosystems, USA)..

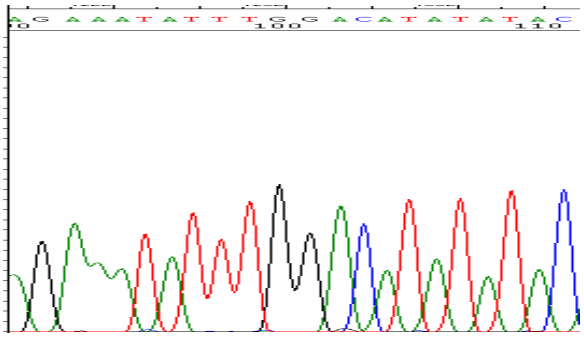

Figure 3.10 Sequencing Analysis software output.

Following the initial analyses, the results are downloaded into the Sequencher software which allows the analysis of multiple samples,

assemble multiple sequences, edit contigs while viewing all relevant trace data, detect and annotate polymorphisms. Figure 3.11.

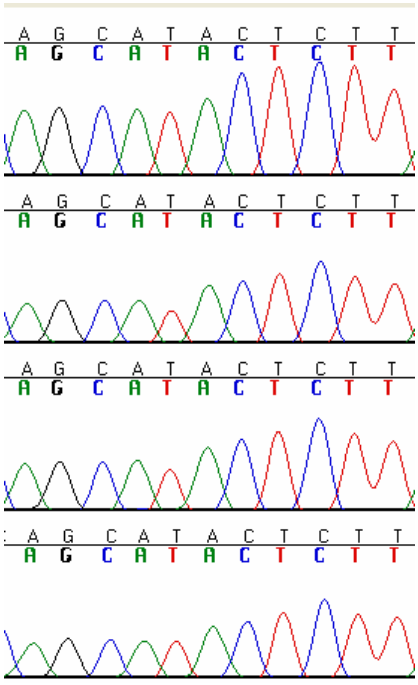

Figure 3.11 Electropherograms of multiple samples aligned with Sequencing software.

To align and compare sequences the free softwares BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and MutationSurveyor were used (Figure 3.12and Figure 3.13).

```
 Score =  825 bits (429),  Expect = 0.0
 Identities = 437/444 (98%), Gaps = 0/444 (0%)
 Strand=Plus/Plus

Query  27   ATTTAGCTGTGCTTATCTAATTTATACATATTTAATTGAAAAACAAAGGCATAACATGAA  86
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  25   ATTTAGCTGTGCTTATCTAATTTATACATATTTAATTGAAAAACAAAGGCATAACATGAA  84

Query  87   TGTCAAGAAATATTTGGACATATATACATCAAACAGCATACTCTTACTAATTTTAATTCC  146
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  85   TGTCAAGAAATATTTGGACATATATACATCAAACAGCATACTCTTACTAATTTTAATTCC  144

Query  147  TGTTAAACAGACTAAAACCTTTTAGTAAAGACCTTTTAGAGTTAAAAATGAATAACCAAT  206
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  145  TGTTAAACAGACTAAAACCTTTTAGTAAAGACCTTTTAGAGTTAAAAATGAATAACCAAT  204

Query  207  ATTTTGCTTAAAATCTAATCTATAGGATGACCTCCCTGGTGGTTCAGAGATTAATATTCT  266
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  205  ATTTTGCTTAAAATCTAATCTATAGGATGACCTCCCTGGTGGTTCAGAGATTAATATTCT  264
```

Figure 3.12 Blast Software ouput.

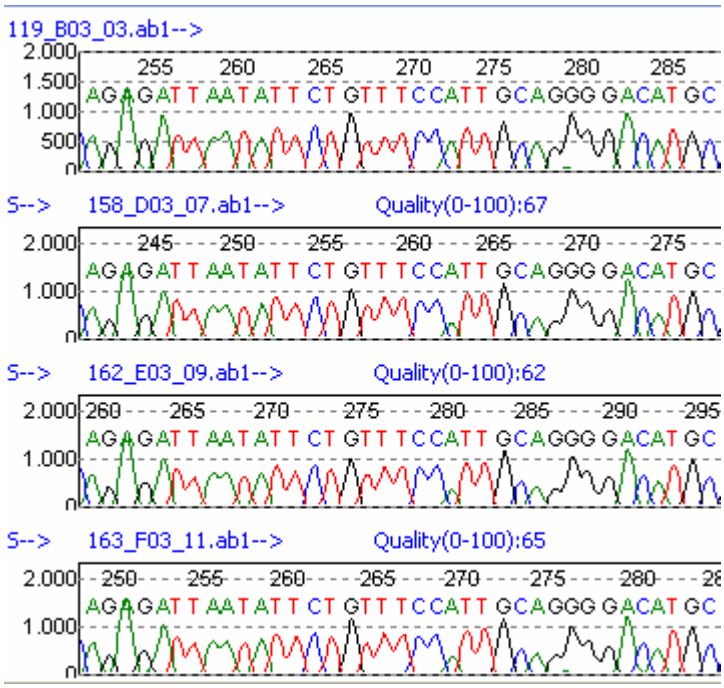

Figure 3.13 MutationSurveyor software output.

3.5. Statistical tools: QTL detection

3.5.1. Interval Mapping

QTL detection, performed with the INRA QTLMap Software, was carried out according to the methodology proposed by Elsen et al. (1999). Usually the interval mapping approach is based on the evaluation of the likelihood on each point along the investigate segment. The population is considered as a set of independent sire families, all dams being themselves unrelated to each other and to the sires. The global likelihood is the following:

$$L^x = \prod_i L_i^x = \prod_i \sum_{hs_i} p(hs_i/M_i) \prod_{j=1}^{n_i} \sum_{q=1}^{2} p\left(d_{ij}^x = q/hs_i, M_i\right) f\left(yp_{ij}/d_{ij}^x = q\right)$$

Where (Lx) is the global likelihood at the point x on the segment; (Lix) is the within sire i likelihood; p(hsi/Mi) is the probability of the sire phase hsi given the family markers information Mi; p(dijx = q/hsi, Mi) is the probability for progeny ij that the allele received from its sire dijx at location x correspond to the qth chromosomes segment including location x (q=1 from the grandsire, q=2 from the granddam) given the sire phase and the family markers

information; f(ypij/dxij=q) is the penetrance function which is conditional on the q chromosome segment transmitted by the sire, ypij is the phenotype of the daughter ij. This penetrance function will be assumed to be normal:

$$f\left(yp_{ij}/d_{ij}^{x} = q\right) = \phi\left(yp_{ij}; \mu_i^{xq}; \sigma_e^2\right) = \frac{1}{\sqrt{2\pi\sigma}} exp\left\{-\frac{1}{2}\left(\frac{yp_{ij} - \mu_i^{xq}}{\sigma_e}\right)^2\right\}$$

Starting from this basic model some modifications are applied in order to reduce the calculation time and to increase the robustness of the method. First, an a priori estimation of the probability of each possible phase of the sires from progeny marker information. Second, a linear approximation of the likelihood. Third, application of a heteroskedastic model considering within-sire family variance different between families.

QTL detection analysis performed in the present work was based on this modified model and was carried out by two steps: (a) for each chromosome, the probability of each possible phase of the sires was estimated from progeny marker information, the most likely phase was retained, and the probability that each progeny received one or the other chromosomal segment was estimated at every position, given this phase; and (b) QTL detection sensu stricto was carried out by within-sire linear regression (Mangin et al., 1999). All positions were tested with a 2 cM step. The linear model was the following:

$$ypij=\mu i-(2*p(dijx = q/his, Mi)*\alpha i/2-1)+eij$$

For each location x on the genome, μi is the fixed effect of the sire; αi is the substitution effect of the putative QTL carried by the sire i and eij is the residual assumed to be normally distributed with a zero expectation and a within-family heterogeneous variance. The residual variance is defined within-sire family to improve detection robustness (Goffinet et al., 1999) and also to simultaneously analyse the traits expressed in different breeds and, possibly, on different scales. In this approach, all parameters (sire and QTL

effects, variances) are defined within-family, and the overall likelihood ratio test is simply the sum of the family contributions.

Because of the multiplicity of the correlated tests along the chromosome, the theoretical distribution of the maximum likelihood test under H0 was unknown and rejection thresholds were estimated by within-family permutations (Churchill and Doerge, 1994), separately for each trait. Ten thousand permutations were computed for each trait and for each permutation the highest likelihood ratio test value over the chromosome was retained. This empirical distribution of tests under H0 was used to define the critical thresholds of 0.05. The number of informative families, i.e. with a sire heterozygous for a detected QTL, was estimated as follows. Assuming that the QTL exists and given its location, there is only one remaining parameter per family, the within sire QTL effect. The sire is considered to be heterozygous at the level if the family contribution to the overall likelihood ratio test exceeded the value 3.84 corresponding to a $\chi 2$ distribution with one degree of freedom and probability 0.05 (Boichard et al., 2003).

The confidence intervals of the QTL location were estimated by 10.000 bootstrapping (Visscher et al., 1996). The empirical sampling distribution of the location was estimated by repeatedly analysing a data set of the same size, randomly sampled within-family in the original data set. Because the distribution of the location of the maximum likelihood under H0 was not uniformly distributed over the chromosome but presented accumulations at the marker location, bootstrap location results were weighed by the inverse of the location frequencies observed under H0, leading to smaller confidence intervals. These location frequencies under H0 were obtained with the permutation results.

3.6. Experimental design to fine mapping the previously found QTL

3.6.1. Backcross population results

To determine the FA content, individual milk samplings at the morning milking were performed twice, in the middle of 2nd (27 March 2001, 847 individuals) and 3rd lactation (26 February 2002, 795 individuals). Two traits were analyzed, the CLA content in the milk fat (mg/g of fat) and the ratio between CLA and vaccenic acid. VA, as already mentioned, is the direct precursor of CLA in the mammary gland where it is desaturated by Δ^9-desaturase to produce rumenic acid.

A repeated measurement model, including as fixed effect, the year of production by group of management interaction and, as random effects, the sire and the animal within sire, was applied prior to QTL analysis. R^2 statistics for the fixed part of the model were calculated for each analysed variable. The random effects were included to provide an estimation of the total individual variance. In particular, the random sire effect was included to permit an evaluation of the additive genetic component, by estimating the variance between half-sib families. Finally, repeatabilities were estimated as the ratio of the sum of sire and animal within sire variances to the total variance. Means and standard deviations were calculated for the analyzed variables, basing on 1454 records of 727 Backcross ewes with two samplings (Table 3-7). The CLA content is consistent with previous results in Sarda sheep (Piredda et al., 2001; Secchiari et al., 2001). Higher values of CLA (32.85 mg/g of fat) were found in Sarda sheep grazing during the spring (Delogu et al., 2000).

On the whole, a remarkable CLA content variability between and within family was detected. Indeed, sire variance was 8.4% of the total phenotypic variance for CLA and 5.6 % for CLA/VA. Total individual variance was 29.7% for CLA content and 34.4% for CLA/VA respectively.

Sire	N° daughters	CLA			CLA/VA		
		mean mg/g of fat	psd	rsd	mean	Psd	rsd
1	74	12.22	3.57	0.73	0.544	0.089	0.030
2	75	12.68	3.65	0.65	0.562	0.090	0.030
3	93	13.42	3.70	0.64	0.567	0.095	0.030
4	75	12.54	3.29	0.78	0.506	0.085	0.029
5	66	11.14	3.20	0.70	0.505	0.075	0.026
6	79	13.14	3.17	0.65	0.530	0.082	0.030
7	66	12.08	3.02	0.75	0.525	0.087	0.034
8	73	12.00	3.20	0.61	0.508	0.074	0.026
9	64	13.14	3.83	0.78	0.537	0.102	0.034
10	62	13.39	3.80	0.71	0.536	0.077	0.026
ALL	727	12.60	3.51	0.70	0.533	0.089	0.030

Table 3-7 Family size, means, phenotypic standard deviation (psd) and individual solution standard deviation (rsd) per family for CLA content and CLA/VA ratio.

Three QTL were detected for the CLA content in milk fat on OAR 4, 14, and 19; four QTL were detected for CLA and VA ratio on OAR 4, 6, 14 and OAR 22 (Table 3-8; Carta et al.2006).

Among the regions affecting CLA/VA ratio, chromosome 22 was chosen for a further investigation because showed a highly significant QTL for conjugated linoleic acid (CLA) and vaccenic acid (VA) ratio that reflects the rate of desaturation of the vaccenic acid in rumenic acid. This QTL is of particular interest also because an evident candidate gene, the SCD gene encoding for Δ^9-desaturase, is located on OAR22. This result can be considered as a first step and suggests that SCD gene polymorphism is actually related to the Δ^9-desaturase expression level and affects the quantity of CLA produced in the mammary tissues from vaccenic acid. This result, indeed, confirms a previous analysis carried out on only the data of the 2nd lactation (Carta et al., 2003). Figure 3.14 shows QTL detection results for CLA and CLA/VA ratio on OAR22.

TRAIT	OAR	Most probable location (cM)	Closest marker	P<	Most informative families id.	Effects
CLA	4	65	MAF50	0.05	6-7-9	0.57-0.47-0.43
CLA	14	56	BM7109	0.01	1-7-8	0.54-0.43-0.49
CLA	19	64	LSCV14	0.01	2-4-8	0.45-0.87-0.33
CLA/VACC	4	72	MAF50	0.05	2-3-6	0.03-0.02-0.03
CLA/VACC	6	12	INRA133	≈ 0.05	4-10	0.02-0.02
CLA/VACC	14	62	BM7109	≈ 0.05	1-2-8-10	0.01-0.01-0.01-.0.2
CLA/VACC	22	54	BM4505	0.01	2-3-4-5	0.02-0.02-0.02-0.02

Table 3-8 Trait, OAR, map position, closest marker, chromosome-wise significant level, families and effects of QTL findings.

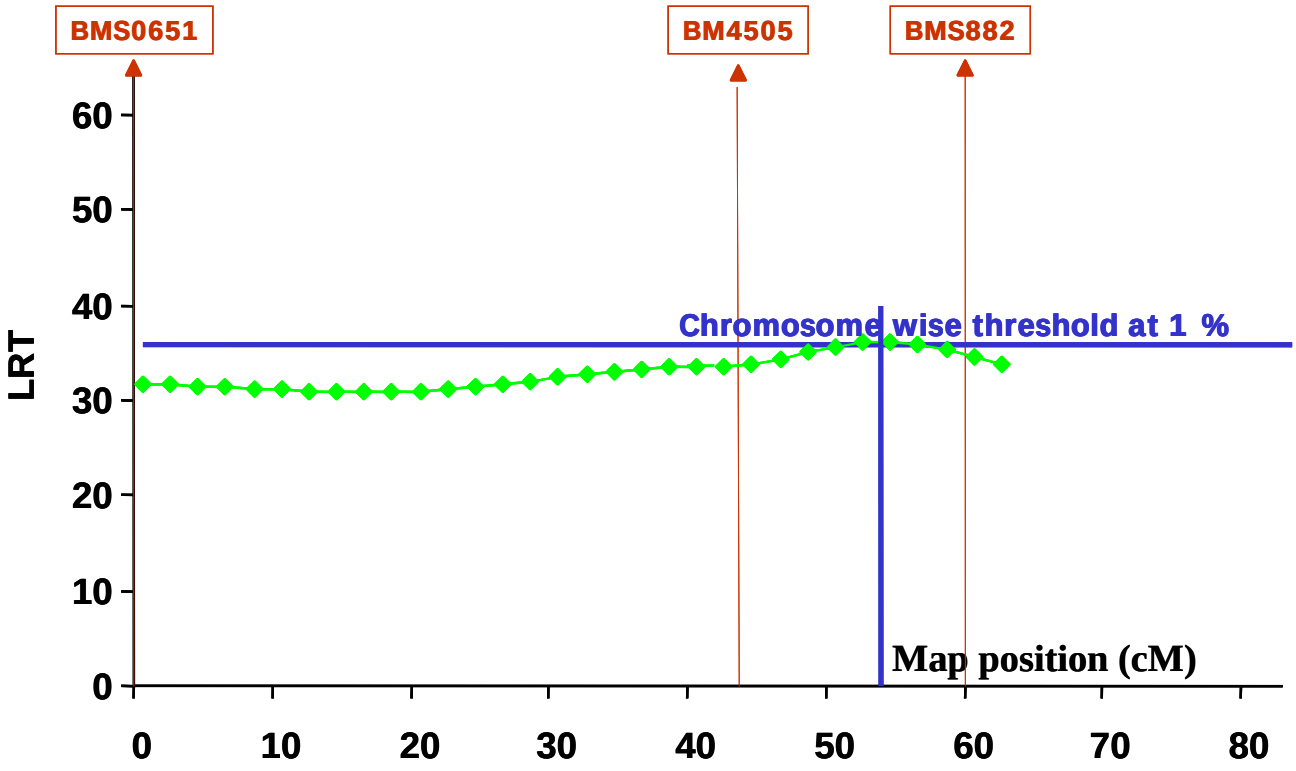

Figure 3.14 QTL results on OAR 22 with 3 microsatellites used for the genome scan.

3.6.2. Building a denser map of the ovine chromosome 22

Fine-mapping on OAR22 was performed adding 7 new microsatellites markers to the 3 used in the previous analysis (Barillet et al., 2005). The microsatellites selected belong to the panel available on Australian Sheep Gene Mapping web site (http://rubens.its.unimelb.edu.au/~jillm/jill.htm) and were chosen basing on marker location, polymorphism content, the good efficiency of amplification and the number of alleles (Table 3-9).

Microsatellite	Primer-(5'-3')	Reference
BMS332	GACAAAACCCTTTTAGCACAGG AATTGCATGGAAAGTTCTCAGC	GenBank Accession: G18841
BMS907	AGTTTCTACTCTGCCACTGTCC TAAAGTCCTGTCTGCCTCTTTC	GenBank Accession: G18751
HEL11	CTTTGTGGAAGGCTAAGATG TCCCACATGATCTATGGTGC	Kaukinen J. & Varvio S.-L.
INRA81	CGGCTCACGGTCTCTATCGG GCGAACCCAAGAATCAGACTC	de Gortari M.J., Freking B.A., Kappes S.M., Leymaster K.A., Crawford A.M., Stone R.T. & Beattie C.W. 1997.
MAF 36	TTGCGAAAAGTTGGACACAATTGAGC CATATACCTGGGAGGAATGCATTACG	Swarbrick P.A., Buchanan F.C. & Crawford A.M. 1991.
TGLA429	TGTACAGTCTGTGGTATAACTTCTT TCTCCTAGAACCTGTCCTCCTAC	--
McM373	GGGTTTACCAGATGTCTGCTTGT TATTTGTCCAGCTGGTTGCAG	Hulme D.J., Smith A.J., Silk J.P. Redwin J.M. & Beh K.J. Genetics 26, 369-370. 1995

Table 3-9 Microsatellites name, primers sequences and reference.

Each locus was amplified using PCR primer pairs fluorescently-labelled with dye 6FAM, PET; VIC or NED and. PCR reactions were carried out in two multiplex (different markers amplified simultaneously in the same vial; Table 3-10).

Multiplex	Microsatellite	Fluorescent label	Size range	Ta (C°)
MP1	INRA81	NED	152-200	58
	HEL11	PET	160-200	
	BMS332	6-FAM	127-157	
MP2	MAF36	NED	99-125	56
	TGLA429	6-FAM	195-213	
	BMS907	PET	80-112	

Table 3-10 Multiplexed microsatellites used in this study. Ta = Annealing temperature.

In 20 µl reaction volume, 50 ng of genomic DNA, 1 X PCR Buffer (KCl 50 mM, TrisHCl 10 mM pH=8.3) ,1.5 mM MgCl2, 200 µM dNTPS, 0.5 U Taq Polymerase and 0.5 µM of each primer were used.

All the reactions were performed using a MJ Research thermalcycler and the thermal profile was:

- ✓ denaturation: 5 minutes at 94°C;
- ✓ annealing: 30 cycles of 30 seconds at 94°C, 30 seconds at 56-58°C (depending on the primer sequence) and 30 seconds at 72°C;
- ✓ extension: 10 minutes at 72°C.

A new QTL detection was done using the method described in 3.5.1 paragraph.

3.6.3. Experimental design to validate the QTL on the 7/8 population

Only markers surrounding the QTL location found in the BC population were genotyped on the 7/8 population with the same reaction conditions described in 3.6.2 paragraph.

A new QTL detection was done using the method described in 3.5.1 paragraph.

3.6.3.1. Fatty acid content analyses

To determine CLA content on the 7/8 population, individual milk samplings at the morning milking were performed in the middle of 2nd lactation (year 2004, 256 individuals; year 2005, 221 individuals; year 2006, 232 individuals).

Phenotypes for QTL detection were individual residuals of a linear mixed model including as fixed effect the interaction between year of collection and lambing month and the father as random effect.

3.6.4. Candidate gene sequencing

Animals from the most significant BC family were sequenced for the one positional candidate gene in order to find causal mutations along this gene: fourteen from the low and fourteen from the high tails of the phenotypic distribution of the ratio between CLA and VA.

Primer pairs to amplify ovine gene were designed using primer 3 software (http://frodo.wi.mit.edu/; Table 3-11). As template to design the primers we used ovine sequence when possible and caprine or bovine sequence when ovine was not available. When possible I preferred caprine sequence for its major homology with ovine one.

To check that no other regions could exist within the exons and the introns sequenced, several overlapping fragments were amplified to screen the regions.

Polymerase chain reaction was carried out in 20 µl of reaction volume, containing 50 ng of ovine genomic DNA, PCR reaction buffer 1X, MgCl2 1.5 mM, dNTPs 0.2 mM, 10µM of each primer and 1U of Taq DNA polymerase

(Euroclone). All the reactions were performed using a MJ Research thermalcycler and the PCR thermal profile was:

- ✓ denaturation: 5 minutes at 96°C;
- ✓ annealing: 35 cycles of 30 seconds at 94°C, 30 seconds at 55-60°C (depending on the primer sequence) and 30 seconds at 72°C;
- ✓ extension: 7 minutes at 72°C.

Amplified products were analyzed by gel electrophoresis on agarose gel (2%) with molecular weight standar and purified using 96 wells MILLIPORE plates (Montage PCR96 Millipore). The protocol includes one filtration step followed by resuspension and recovery of the sample in a volume of 25µl of water.

Amplified products were then sequenced in both directions with ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction v.3.1 (Applied Bio-systems, Foster City CA, USA) according to the supplier's protocol with some modifications. The sequence thermal profile was:

- ✓ denaturation: 10 seconds at 96°C;
- ✓ annealing: 25 cycles of 5 seconds at 55-64°C;
- ✓ extension: 4 minutes at 60°C.

The unincorporated dye terminators and salts were removed from sequencing products using 96 wells MILLIPORE plates (Montage SEQ 96 Millipore) to generate high quality DNA.

Primer name	Prime (5'-3')	size (bp)	Ta	Origin
Promoter 1	cacctgcccagacttctctc gacctcactgttgtggctca	360	60°	bovine
Promoter 2	aatcccaggtgcctctcttc gtcacagcagccaatcaatg	300	60°	bovine
Promoter 3	cctgggctccatcattacat gggtcgttgttttggaattg	250	58°	bovine
Promoter 5	ggacacgtctcccctcctac caatctgctgttccctctgc	200	58°	bovine
Promoter 6	gcagagggaacagcagattg gggtgactgtgtcccgtatt	240	55°	bovine
Promoter 7 – 5'UTR-exon 1	ctctgtctcctcccctctcc gctctcacctcctcttgcag	285	64°	bovine
Exon 2	tcctctctcttgaccctcca gggaggacatgggaacttct	300	60°	caprine
Exon 3	gaaacacttccccaggtgag tggactttgggactgaacct	265	60°	caprine
Exon 4	gaattcccctgagagggtgt atatagaaaccaagtaaatttctcagc	305	60°	caprine

Primer name	Prime (5'-3')	size (bp)	Ta	Origin
Exon 5	atttcttgcccattccctct cccagggaaaccaggatatt	240	58°	caprine
Exon 6	cattctgctagggaaccagag caagggaccagaaactcagc	225	62°	caprine
Intron 1A	caaaagcaggctcaggaact cctcaaggtgaaggggaaat	539	58°	ovine
Intron 1B	ttcctttgtgtgctcgtttg acagcgctcccaagtgtaac	660	58°	ovine
Intron 2B	ggaggggacttgggagaata tgatcaggaagacccgtagg	320	60°	ovine
Intron 3	tggagtcaccgaacctacaa gaattgtggggatcagcatc	1425	60°	ovine
Intron 4A	cgacgtggctttttcttctc ccttccactccccaataagc	360	60°	ovine
Intron 4B	gacgtaggacggtcatggat cacaacagcgtaacggagaa	660	58°	ovine
Intron 5A	ttctccgttacgctgttgtg ggcgacacagtcaaaagtca	450	60°	ovine
Intron 5B	cccaggtgaaagctcaaaga ggtagttgtggaagccctca	570	60°	ovine
3UTR A	aaggcaagcagttggtcagt tcacagctatttcccagagtca	450	60°	ovine
3UTR B	aaggcaagcagttggtcagt tcacagctatttcccagagtca	460	60°	ovine

Table 3-11 Primer name, primers sequence, products size, annealing temperature (Ta) and template used to design the primers origine's.

4. Results

4.1. QTL detection on the backcross population with the denser map

The four families of the BC population showing significant contrasts in the previous analysis (Table 4-1; Carta et al., 2003a, 2006) were genotyped for the new microsatellites.

Sire	QTL effect	test t	LRT contribution
1	-0.85	-1.33	1.75
2	1.82	2.71	7.06
3	1.34	2.10	4.33
4	-2.13	-3.34	10.62
5	2.15	3.19	9.60
6	0.48	0.71	0.51
7	-0.27	-0.32	0.10
8	-0.12	-0.15	0.02
9	-1.56	-1.51	2.25
10	0.22	0.29	0.08

Table 4-1 Sire, QTL effect, test t results, LRT contribution and in bold type the four significant families.

Respect to the original 3 microsatellites used in the genome-scan, the addition of 7 new markers led to an increase of the explored chromosome segment's size to 100% of the total length of chromosome. A specific genetic map was built using the whole population. The ten analysed microsatellites are reported in Table 4-2. BMS0651 was not included in the linkage group by CRIMAP software due to the fact that no sire was double heterozygous at the BMS0651 and one of the two following marker loci. The average distances between loci (map's density) was 17,5 cM in the denser map but in the fragment around 35 cM the average distances between markers was 4,5 cM (Figure 4.1).

No remarkable difference from the Sheep Best Position Linkage Map Version 4.4 (sex averaged, cM), available at the Australian Sheep Gene Mapping web site, was detected then it was retained for the QTL analysis. The average information content (AIC) over the whole chromosome, calculated as the mean of $|2pij- 1|$ on the four analysed families, is reported in Figure 4.1. It ranged from 0.76 at the beginning to 0.98 at the end of the analysed

segment. On the whole, the marker density and the information content were high.

The new QTL detection analysis (Carta et al., 2006) highlighted the presence of one highly significant QTL with the most probable location at 35 cM (Figure 4.1), corresponding to the position of the SCD gene on the available maps (34.5 cM Maddox, Australian Sheep Gene Mapping Web Site 2007), with LRT value of 57.1. After 100,000 permutations the chromosome–wise probability of no QTL resulted lower than 0.00001 (highest LRT under H0 of 51.1). The QTL substitution effects ranged from 0.5 to 1 residual standard deviation units. The 95% bootstrap confidence interval was between 31 and 55 cM. (68.5 % of locations between 31 and 36 cM).

Chromosome	Microsatellites	P A	Family	n° genotypes	informative meiosis
22 (83cM)	BMS0651	0	all	963	331
	BMS907	13.8	2;3;4;5	354	229
	HEL11	30	2;3;4;5	372	342
	INRA81	35.7	2;3;4;5	368	260
	BMS332	36.9	2;3;4;5	353	289
	BM4505	43.5	all	971	645
	BMS882	59.7	all	971	514
	TGLA429	62.1	2;3;4;5	553	439
	MAF36	77.2	2;3;4;5	371	340
	McM373	82.9	2;3;4;5	951	872

Table 4-2 Chromosome dimension in the Australian Sheep Gene Mapping (ASGM), microsatellites, position in ASGM, n° of genotype and n° of informative meiosis; in bold type: densification markers; normal type genome scan markers.

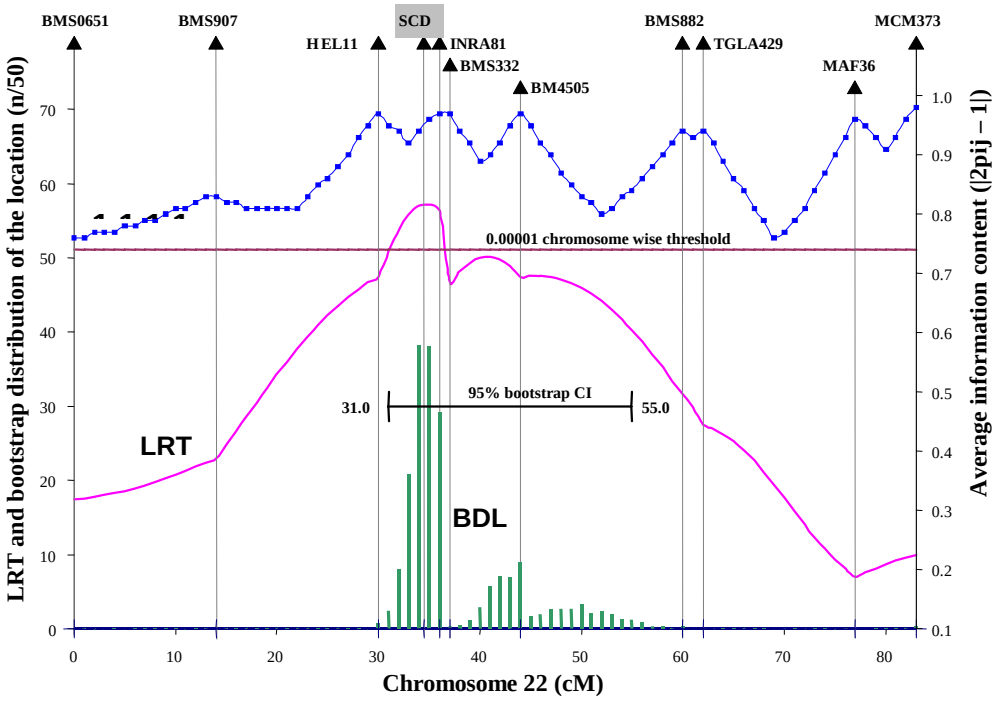

Figure 4.1 Likelihood ratio test profile (LRT), average information content (AIC) and bootstrap distribution of locations (BDL).

4.2. QTL detection on the 7/8 population

The aim of analyzing the 7/8 population was validating in the Sarda pure breed the QTL found in the BC population by verifying the QTL segregation in the 7/8 families, although this population structure may allow detecting a QTL independently from the results of the BC population.

Means and standard deviations were calculated for the analyzed variables, basing on 644 single individual records (Table 4-3).

Sire	N° daughters	CLA			CLA/VACC		
		mean mg/g of fat	Psd	rsd	mean	psd	rsd
1	37	15,226	2,719	2,626	0,441	0,053	0,051
2	22	18,852	2,894	2,556	0,540	0,087	0,067
3	34	16,476	3,127	2,873	0,536	0,141	0,116
4	19	18,555	2,864	2,807	0,507	0,087	0,075
5	36	23,228	9,192	9,128	0,659	0,179	0,097
6	33	18,296	4,501	4,562	0,686	0,649	0,101
7	83	21,618	6,628	6,362	0,658	0,144	0,146
8	40	15,667	3,461	3,425	0,422	0,064	0,055
9	44	21,578	5,378	5,303	0,579	0,090	0,085
10	35	13,745	2,682	2,752	0,428	0,055	0,057
11	49	14,911	3,107	3,118	0,453	0,067	0,071
12	35	20,314	7,771	7,519	0,564	0,088	0,089
13	25	16,228	3,120	2,926	0,452	0,078	0,080
14	28	16,473	3,481	3,465	0,511	0,083	0,081
15	26	18,980	6,176	6,488	0,625	0,129	0,130
16	28	22,637	4,710	4,663	0,586	0,093	0,087
17	37	19,103	4,107	4,135	0,566	0,101	0,099
18	33	18,747	6,874	6,754	0,570	0,105	0,103
ALL	644	18,546	5,849	5,061	0,550	0,196	0,096

Table 4-3 Famly size, means, phenotypic standard deviation (psd) and individual solution standard deviation (rsd) per family for CLA content and CLA/VA ratio on the 7/8 experimetal population.

The analysis on the 7/8 population was realized only for four markers surrounding the QTL location found in the BC population (HEL11, INRA81, BMS332, BM4505 Table 4-4).

Chromosome	Microsatellites	Analyzed Families	Tototal genotypes	Useful genotypes	Informative meiosis
22 (83cM)	HEL11	All	776	773	714
	INRA81	All	794	781	718
	BMS332	All	793	789	503
	BM4505	All	796	790	435

Table 4-4 Chromosome (dimension in the international map), microsatellites, analyzed families, n° of total genotypes, n° of useful genotypes and n° of informative meiosis; bold type: densification marker on the 7/8 population.

QTL segregated in families 2, 6 and 10 with LRT within family ranging from 4.50 to 13.69 (Table 4-5).

Family	QTL effect	t Test	LRT contribution
1	-1.78	-1.08	1.15
2	-8.51	-4.11	13.69
3	0.10	0.03	0.00
4	3.81	1.39	1.87
5	0.72	0.23	0.05
6	6.23	2.17	4.50
7	-0.52	0.16	0.02
8	0.53	0.31	0.09
9	-1.47	-0.54	0.29
10	3.70	2.22	4.65
11	0.30	0.14	0.02
12	0.15	0.06	0.00
13	1.27	0.39	0.16
14	-2.41	-0.75	0.56
15	4.53	0.96	0.91
16	5.50	1.57	2.38
17	3.25	1.00	0.98
18	-3.99	-1.21	1.44

Table 4-5 Sire, QTL effect, test t results, LRT contribution and in bold type the three significant families of the 7/8 population.

4.3. Biopsys

In order to carry out expression study, mammary biopsys sampling were achieved from some animals from the most significant 7/8 family (Table 4-5).

4.4. Detecting and sequencing one positional candidate gene

Ovine map is not very informative but detailed human/bovine comparative maps have been developed (Hayes 1995; Band et al. 2000; Hayes et al. 2003, Gautier et al 2003), increasing the possibility of exploiting the genome sequence and the growing functional characterization of reference species such as man (Lander et al. 2001), rat (Gibbs et al. 2004), or mouse (Waterston et al. 2002). These data provide new insights to unravel the genetic determinism involved in the variation of some traits of breeding interest (Andersson and Georges 2004).

Several genes involved in lipid metabolism or catabolism pathways are located on Bos Taurus 26, homologous of Ovis Aries chromosome 22. They all were *a priori* strong functional candidate genes. Among this we can quote LIPF (gastric lipase), LIPA (lipase A, cholesterol esterase), SCD (stearyl co-A

desaturase), and GPAM (glycerol-3-phosphate acyltrans-ferase, mitochondrial). As aforesaid, the most likely position of the QTL was found to be located in the marker bracket Hel11/INRA81 and this region contains only one characterized gene by direct mapping in ovine and by comparative mapping in bovine (Figure 4.2): SCD gene that represents a strong positional candidate gene since it is a key enzyme responsible for Δ^9-desaturation of fatty acids in the ovine mammary gland and other tissue.

This result supported the sequencing of this gene to find causal mutations.

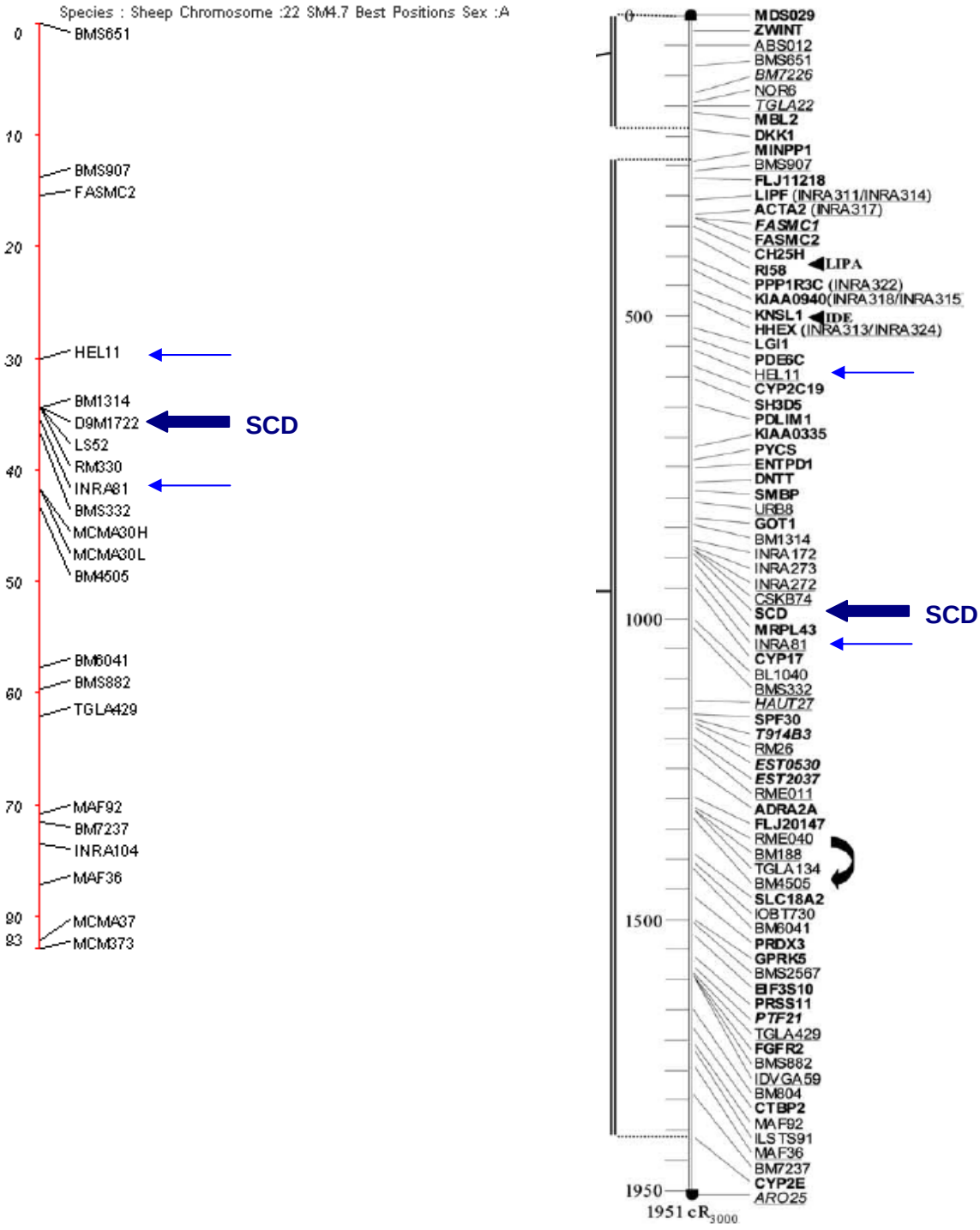

Figure 4.2 Ovis Aries chromosome 22 (Maddox, Australian Sheep Gene Mapping Web Site: Sheep Best Positions Linkage Map Version 4.7, 2006) and Bos Taurus chromosome 26 (Gautier et al. 2006 Radiation Hybrid Map): genes and markers.

4.4.1. Sequencing the SCD ovine gene

The sire and twenty-eight ewes belonging to the most significant family (Table 4-6 Likelihood Ratio Test=29.13) were selected to sequence the SCD gene: fourteen from the low tail and fourteen from the high tail of CLA and VA ratio phenotypic distribution (Table 4-7).

Family	N° of Daughters	CLA/VA mean% (mg/g of fat)	CLA/VA r.s.d.%	QTL effect	t Test	LRT contribution
1	74	54.4	3.0	-0.84	-1.19	1.41
2	75	56.2	3.0	1.95	3.09	9.10
3	93	56.7	3.0	1.55	2.70	7.08
4	75	50.6	2.9	-1.93	-3.50	11.64
5	66	50.5	2.6	3.08	5.91	29.13
6	79	53.0	3.0	1.11	1.46	2.10
7	66	52.5	3.4	0.28	0.28	0.08
8	73	50.8	2.6	-0.08	-0.09	0.01
9	64	53.7	3.4	-0.66	-0.52	0.27
10	62	53.6	2.6	0.13	0.14	0.02

Table 4-6 Means, phenotypic s.d (psd) and individual solution s.d. (rsd) per family for CLA content and CLA/VA ratio and in bold type the four significant families.

Animal	**Phenotypic value**	**Animal**	**Phenotypic value**
812	-5,284	709	1,5207
629	-4,777	388	1,5267
19	-4,605	754	1,6411
394	-4,403	843	2,2466
856	-4,239	481	2,4952
195	-4,126	348	2,8971
344	-4,005	572	3,4748
846	-3,355	872	3,6090
483	-3,211	283	4,3797
620	-3,032	970	4,5688
677	-2,680	965	4,7217
578	-2,325	22	4,7928
881	-2,106	607	5,1657
891	-0,898	493	6,2866

Table 4-7 Low and high tail of the phenotypic distribution of the ratio between CLA and VA .

The SCD sequence displayed initiation and stop codons, defining the coding region, so, finally, the whole coding region (6 exons), the promoter region, the 5'-UTR and 3'-UTR were sequenced. Moreover introns 1, 3, 4 and 5 were completely sequenced whereas some portions of intron 2 and promoter are still to sequence (Miari et al. 2007).

Only ovine SCD coding region was reported in literature (Ward et al. 1998, GenBank accession n° NM_001009254), so the alignment of SCD sequences was done using caprine SCD gene like reference. In fact GenBank database search using Blast (Altschul et al., 1997) revealed a high degree of similarity between the ovine SCD coding region and those of goat (98%), cattle (95%) and pig (90%), whereas it is lower with those of man, rat and mouse (SCD-1 and -2).

4.4.2. Characterization of the ovine SCD promoter

Results of Keating et al. (2005) indicate that the 407 bp region upstream of the transcription start site is sufficient to direct transcription. The high conservation of this region may be significant in indicating that the regulation of this gene is under extremely rigid control by transcription factors and high conservation is necessary for full activation. We sequenced 374 bp of this region and, according with Keating et al 2005, in the comparison of ovine, bovine and human sequences we identified a number of conserved sequences in two particular regions of the promoters (Figure 4.3).

In the first area of conservation occurs there are two conserved TATA sequences (5'-TTTAAAT-3' and 5'-TAAAA-3'), a fat-specific element (FSE) (5'-CTGAGGAAA-3'), and binding sites for the transcription factors AP-1, NF-1, and HNF4. The TATA sequence, TTTAAAT, is somewhat unusual: this sequence where the A in the second position is replaced by a C, T, or G, has been shown to reduce the efficiency of a promoter in vitro studies (Conchino et al. 1983).

```
          RFX1                NF-1                  NF-Y/AP1
HUM       GCAACGGCAG GACGAGGTGG CACCAAATTCC CTTCGGCCA ATGACGA
BOS       GCAACGGCAG GACGAGGTGG CACCAAATTCC CTTCGGCCA ATGACGC
OVI       GCAACGGCAG GACGAGGTGG CACCAAATTCC CTTCGGCCA ATGACGC


HUM       GCCGGAGTTT ACAGAAGCCT CATTAGCATTT CCCCAGAGG CAGGGGCAGG
BOS       GCCAGAGTCT ACAGAAGCC: CATTAGCATTT CCCCAGGGG CAGGGGCAGA
OVIS      GCCAGAGTCT ACAGAAGCC: CATTAGCATTT CCCCAGGGG CAGGGGCAGA


HUM       GGCAGAGGCC G:GGTGG:TG TGGTGTCG:GT GTCGGCAGC ATCCCCGGCG
BOS       GGCAGGGGCT GCGGCGGCCA AGCCGCGGTGT GTGTGCAGC ATCCAGTTCT
OVIS      GGCAGGGGCT GCGGCGGCCA AACCGCGGTGT GTCTGCAGC ATCCAGTTCT


HUM       CCCTGCTGCG GTCGCC:GCG AGCCTCGGC:C TCTGTCTCC TCCCC:CTCC
BOS       TGCTTCTTCG GCCCCCAGCA CGCCTCGGCGC TCTGTCTCC TCCCCTCTCC
OVIS      CGCTTCTCCT GCCCCCAGCA CGCCTCGGAGC TCTGTCTCC TCCCCTCTCC

                                                              HNF-4
HUM       CGCCCTTACC TCCACGCGGG ACCGCCCGCGC CAGTCAACT CCTCGCACTT
BOS       CGCCC::A:: T::GCG:GAT CTCCCACG:GT GAACCAACT CTGCGCACTT
OVIS      CGCCC::A:: T::GCG:GAT CTCCCACG:GT GAGCCAACT CTGCGCACTT

                NF-1        TATA sequence      FSE
HUM       TGCCCCTGCT TGGCAGCGGA TAAAAGGGGGC TGAGGAAAT ACCGGACACG
BOS       TGCCCCTTGT TGGCAACGAA TAAAAGAGGTC TGAGGAAAT ACGGGACACA
OVIS      TGCCCCTTGT TGGCAGCGAA TAAAAGGGGTC TGAGGAAAT ACGAGACACA

          AP-1                  TATA sequence
HUM       GTCACCCGTT GCCAGCTCTA GCCTTTAAATT CCCGGCTCG GGGACCTCCA
BOS       GTCACCCCCT GCCAGCGCTA GCCTTTAAATC CCCAGC::: ::::::::::
OVIS      GTCACCGGCT GCCAGCGCTA GCCTTTAAATC CCCAGC::::::::::::::

                                          +1
HUM       CGCACCGCGGCTAGCGCCGACAACCAGCT:AG
BOS       ::ATAGCAGGTCGGGTCCGGACACCGGTCCAG
OVIS      ::C:AGCAGGT:CG:GGTCCGGACCGATCCAG
```

Figure 4.3 Sequence aligment of Human (AF320307) and Bovine sequence (AY241932) with sequences Scd1 promoter of this study. In figure are indicated in bold type two TATA sequence, binding sites for the transcription factors (AP-1, NF-1, HNF-4), a fat-specific element (FSE). Human transcription start site is indicated by +1. RFX1 is X-box-binding regulatory factor.

4.4.3. Comparison of ovine, bovine and caprine SCD intron 3

An alignment of ovine intron 3 sequence was performed with a bovine (AY241932) and caprine (AH011188) intron 3 sequence. Only the first part of intron 3 (about 735 bp) exhibits, as expected, a high homology (about 97%) with caprine whereas the second part (about 813 bp) is absent in the caprine sequence and is highly homologues with the bovine one (about 91%).

All the other ovine SCD sequences exhibit big homology with caprine sequences (from about 95% to 99%).

4.4.4. Polymorphism of the ovine SCD gene

I sequenced about 9 kb of the ovine SCD genomic DNA and one single nucleotide polymorphism (SNP) was identified at intron 4 (3295 C>T in Capra hircus stearoyl coenzyme A desaturase gene, GenBank accession n° AH011188).

The sire resulted heterozygous C/T. Out of the 14 animals of the high tail, 13 inherited the T allele (C/T genotype) and the positive marker haplotype (Table 4-8) whereas only one animal inherited the C allele (genotype CC) and the negative marker haplotype. Out of the 14 animals of the low tail, 13 inherited the C allele (C/C genotype) and the negative marker haplotype whereas only one animal inherited the T allele (C/T genotype) and the positive marker haplotype (Table 4-9). Figure 4.4 shows the SNP's point electropherogram.

High tail animals	Microsatellites		
	HEL11	INRA81	BMS332
709	176		140
388	176		146
754	176	152	146
843	176	152	140
481	176	152	
348	170	176	146
572	176		140
872	176	152	140
283	176	152	146
970	176	152	
965	176	152	140
22		152	140
607	176	152	140
493	176	152	

Table 4-8 High tail animals with positive marker haplotype. In bold type the animal with high CLA/VA ratio and negative marker haplotype. Blank indicates that it was not possible to identify the marker allele received by the sire.

Low tail animals	Microsatellites		
	HEL11	INRA81	BMS332
812	170	176	146
629	170	176	146
19	170		146
394	170	176	146
856	170	176	146
195	170	176	146
344	170	176	146
846	176	176	146
483	170	176	146
620	176	152	146
677	170	176	
578	170	176	146
881	176	176	
891	176	176	140

Table 4-9 Low tail animals with negative marker haplotype. In bold type the animal with low CLA/VA ratio and positive marker haplotype. Blank indicates that it was not possible to identify the marker allele received by the sire.

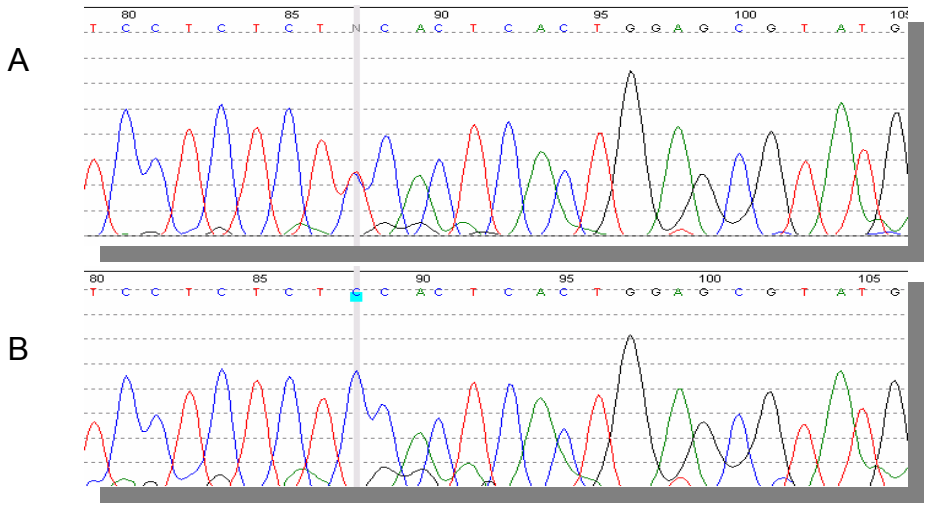

Figure 4.4 Electropherogram of two samples: A is a sample from the high tail of CLA/VA distribution (heterozygous C/T) and B is a sample from the low tail of CLA/VA distribution (homozygous C/C); the grey line shows polymorphism point.

Then, also the 3 sires of the other three significant families (Table 4-6) were sequenced for the whole SCD gene. No other polymorphism was identified and they resulted homozygous C/C at the SNP found on the first family. The 18 sires of the 7/8 Sardinian x Lacaune population (Table 3-4) were sequenced only for a small gene portion surrounding the found SNP. They resulted homozygous C/C.

5. Discussion and conclusion

Selection for CLA content in sheep milk fat is important to enhance the nutritional value of sheep milk cheese without negatively affecting the typical taste and flavour. Several studies have highlighted the importance of nutrition in modifying the FA profile either in cattle or sheep and goats (Demeyer and Doreau, 1999; Chilliard et al., 2000). Moreover, many reports are available on the effects of different pastures on FA profile of sheep milk (Cabiddu et al., 2005; Addis et al., 2006). By contrast, many fewer studies are available on the genetic determinism of these traits. This is mainly due to the fact that large-scale phenotyping is needed to produce accurate genetic studies and, that until recently, the only available technique has been very time-consuming and costly gas chromatography technique. However, Soyeurt et al. (2006a) showed that genetic variation exists across and within cattle breeds for FA profile of milk, using a mid-infrared spectrometry technique which was less laborious and expensive. In sheep, Carta et al (2008) basing on the backcross Sardinian x Lacaune population data, estimated repeatabilities of the most important FA and their ratios or sums. All the analysed variables showed important individual variabilty. These results suggest that a certain amount of additive genetic variance is available for selection purposes for most FA. This was further confirmed by the values of the estimated sire variances, which represents the proportion of variability attributable to genetic differences between sires. In this study, however, it has to be recognised that sire variances values could be inflated by other sources of covariation between daughters of the same sire. In fact, the experimental population was created by mating each F1 sire with ewes homogeneous for age and flock of origin, and each half-sib family was managed in a homogeneous way. From a selection point of view, these results indicated that most of the variables could be selected by the classical quantitative approach. This strategy would require the feasibility of large-scale phenotyping in the registered flocks at reasonable costs. As previously mentioned, the costs of the milk sampling and gas chromatography analyses are high. The difficulty of a large-scale phenotyping is even more evident in sheep where the individual recording of any trait is much more costly

compared to dairy cattle due to the low individual income from each ewe. However, the recent applications of a mid-infrared technique for FA content determination in cow milk seem to be promising for the implementation of this strategy in dairy sheep also. However research combining classical quantitative approaches and quantitative trait loci detection is needed, either on-farm, by implementing experimental recording schemes, or in experimental flocks.

This thesis is included in the framework of a general research aimed at finding marker haplotypes or genes involved in the genetic determinism of traits difficult to select with the classical quantitative approach. Specifically, I was interested in exploring the possibility of implementing genome information based selection approaches for CLA content in sheep milk fat. Carta et al (2008) showed a high number of QTL affecting the most of FA and their combinations or ratios. Starting from these results, my study highlighted the presence of a highly significant QTL on the ovine chromosome 22 affecting the rate of desaturation of vaccenic acid to conjugated linoleic acid in the mammary gland of sheep. The results of the validation of this QTL in the Sardinian pure breed population indicated that this QTL is probably segregating also in the Sardinian population even if in a small percentage of families. In this QTL region the positional candidate approach led to identify a strong functional candidate gene: the SCD gene, coding for the Stearoyl-coenzyme A desaturase, a key enzyme responsible for Δ^9-desaturation of fatty acids in the ovine mammary gland and other tissue. The sequencing of a large part of this gene allowed to identify only one single nucleotide polymorphism (SNP) at intron 4 (3295 C>T, Miari et al. 2007). This mutation was recovered only on one significant family while it was absent in the other significant families and in all Sardinian pure breed sires. Moreover, the found SNP maps in a non-coding sequence region and then it does not produce a structural change in the SCD enzyme.

In conclusion, the overall results of our study did not provide a clear indication that the found mutation is the causal one. Therefore, this study must be completed by sequencing the remaining part of the SCD ovine gene

(a promoter portion and a intron 2 portion), to ascertain if there are additional SNPs linked to the SNP found in this study, and if these SNPs have a structural or regulatory role. In particular it is essential the complete sequencing of the promoter region since it might affect gene expression.

Whether the complete sequencing will confirm that the mutation found is the only one present, further studies will be necessary. Expression studies should be carried out to seek cDNA differences. Subsequently others candidate genes located on Ovis Aries chromosome 22 will be investigated. Bos Taurus chromosome 26 comparative mapping results indicates that several genes involved in lipid metabolism or catabolism pathways are located on BTA26 (Gautier et al 2006). Among these, from the chromosome centromere to the telomere we can quote LIPF (gastric lipase), LIPA (lipase A, cholesterol esterase), or GPAM (glycerol-3-phosphate acyltransferase, mitochondrial).

As a whole, this study confirms that the strategy of saturating the genetic maps of QTL regions, identified by genome scan approaches based on intermediate density genome maps, can actually help to identify positional candidate genes or a set of strongly linked markers haplotypes.

Although this study did not allow for the immediate application of Marked Assisted Selection or Gene Assisted Selection for CLA content in sheep milk fat, the high significance of the found QTL clearly indicates that this is a very crucial genome region. Basing on this result, the ongoing research are expected to identify a strongly linked markers haplotype or a causative mutation to use for Marked Assisted Selection or Gene Assisted Selection. With this perspective, it must be kept in mind that both correlation estimates and pleiotropic QTL findings by Carta et al (2008) highlighted that the fatty acid profile of sheep milk fat is a very complex system resulting from numerous interrelationships between basic fatty acids. This suggests that strategies for improving fatty acid composition have to take into account that increasing one specific fatty acid could lead to changes of the whole profile whose effects on nutritional value of milk should be monitored.

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