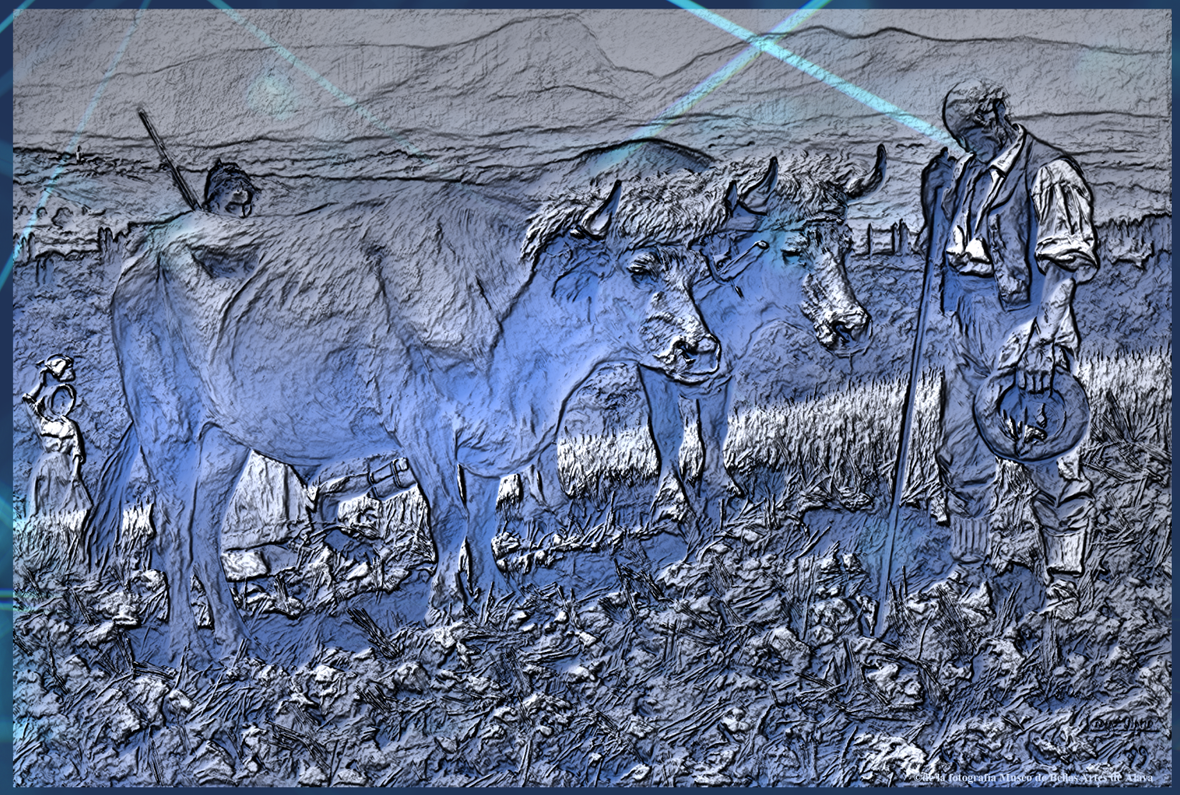
GENETIC CHARACTERIZATION AND GENE EXPRESSION ASSOCIATED WITH FATTY ACID PROFILE IN CATTLE

CARACTERIZACIÓN GENÉTICA Y EXPRESIÓN GENICA ASOCIADA AL PERFÍL DE ÁCIDOS GRASOS EN GANADO VACUNO

BEHI-AZIENDAREN KARAKTERIZAZIO GENETIKOA ETA GANTZ AZIDOEN PROFILARI LOTUTAKO ADIERAZPEN GENIKOA

Ph. D. Thesis | Tesis Doctoral | 2022
David Gamarra Fernández



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RESUMEN

Esta Tesis Doctoral se ha realizado en colaboración entre los Grupos de Investigación BIOMICs y Lactiker de la Universidad del País Vasco (UPV/EHU), que se dedican a la investigación multidisciplinar en el campo de la genética, y la calidad de los alimentos de origen animal, respectivamente.

El sector agroalimentario es de gran importancia para la economía europea. De hecho, la Unión Europea (UE) es el mayor exportador mundial de productos agroalimentarios, siendo España el tercer productor de la UE. Dentro de España, en la Comunidad Autónoma del País Vasco (posteriormente País Vasco), el sector bovino supone el principal valor económico de la producción ganadera. A su vez, el sector bovino está estrechamente ligado al estilo de vida vasco tradicional y al pastoreo. En relación a la producción de alimentos, la nueva política europea en reproducción animal está marcando una tendencia hacia sistemas de producción sostenibles utilizando recursos genéticos animales de origen autóctono. Para ello, es necesaria una gestión eficaz de los recursos genéticos animales, exigiendo un conocimiento claro de las características de cada raza, lo que implica conocer la distribución geográfica, el sistema productivo, la diversidad genética intra e interracial y los datos poblacionales, entre otros. Igualmente, la selección genética es una estrategia en continuo desarrollo orientada a la mejora de la producción en respuesta a los nuevos mercados, siendo una herramienta eficaz para la mejora de la calidad de la carne. Es bien conocido que la existencia de variabilidad genética en toda raza animal, por tanto, su estudio y comprensión en relación con características de la calidad de la carne ha supuesto un paso necesario hacia la selección asistida por marcadores.

Los principales objetivos de esta Tesis Doctoral han sido (1) la caracterización genética de varias razas bovinas bien establecidas en el País Vasco para el análisis de la diversidad genética, estructura poblacional, efecto fundador, alelos asociados a migraciones históricas, eficiencia de varios paneles de STRs en asignaciones genéticas, y estudio de las relaciones filogenéticas con otras razas bovinas europeas; y (2) caracterizar la composición de ácidos grasos (AG) del tejido adiposo de

diferentes tipos comerciales (obtenidos a partir de varias razas producidas en el País Vasco) con el objetivo de asociar este perfil de AG a la expresión génica y a polimorfismos de genes lipogénicos.

La genética se ha convertido en una poderosa herramienta para la caracterización de los recursos genéticos animales y un método práctico en el estudio de la diversidad de especies animales y sus razas. Igualmente, los microsatélites (STR) han mostrado ser biomarcadores genéticos eficaces para exclarecer casos de identificación individual y parentesco biológico en animales. Para afrontar el primer objetivo, las metodologías analíticas aplicadas pasaron desde la PCR convencional, al análisis de fragmentos por secuenciación mediante electroforesis capilar. Para empezar, se evaluó la eficacia de un panel comercial de 12-STRs en poblaciones bovinas de Terreña y Salers para casos de parentesco y trazabilidad biológica. Los resultados mostraron que los valores del Poder de Exclusión Combinado (CPE) estaban directamente relacionados con el número de loci analizados, y concluyeron que este conjunto de marcadores era suficiente para la identificación correcta de un individuo. Sin embargo, se recomienda aumentar el número de marcadores STR para garantizar una paternidad biológica acreditada.

La raza Salers, originaria de Francia, fue introducida en el País Vasco y se ha expandido a otras regiones de España como respuesta a la demanda de una rápida especialización en la gestión ganadera mediante el uso de razas alóctonas altamente productivas. En el segundo estudio, se ha usado el panel comercial de 12-STRs, previamente utilizado, para la caracterización genética y la evaluación de un posible efecto fundador en la población de Salers de España en relación a un bajo número de reproductores iniciales. La población de Salers reveló la ausencia de un efecto fundador en la expansión de esta raza en España, cuando se estudió su población utilizando Pruebas de Signo y Pruebas de los rangos con signo de Wilcoxon bajo modelos de mutación por pasos (SMM) y de mutación en dos fases (TPM). Estos análisis mostraron que la población de Salers parece estar en equilibrio de mutación-deriva, debido probablemente a una incorporación constante de animales reproductores a esta población.

La raza de ganado autóctono Pirenaica es de gran importancia como recurso genético animal local y su carne está registrada bajo el amparo europeo de calidad como

indicación geográfica protegida (IGP) del País Vasco. En base a nuestros primeros resultados, se recomendaba aumentar el número de marcadores STR analizados. Por tanto, como siguiente paso, se ha investigado la eficiencia del panel de 30-STR, recomendado por la ISAG/FAO, para su uso en casos de parentesco biológico, identificación individual y para la selección de los marcadores más discriminantes en Pirenaica y otras razas típicamente criadas en el País Vasco (Terreña, Blonda de Aquitania, Limusina, Salers, y Holstein o Frisona). Estos resultados revelaron que es necesario un panel mínimo de 21-STR tanto para un resultado de parentesco biológico fidedigno en ausencia de información genética de uno de los progenitores, como para realizar asignaciones genéticas fiables basadas en modelos predictivos de machine-learning (aprendizaje automático).

En general, la caracterización genética de la razas autóctonas Pirenaica y Terreña, y la raza alóctona Salers, ha revelado la presencia de alelos diagnósticos de cebú africano (BM2113-123, BM2113-131, BM2113-143 y ETH152-193). En Salers, estos alelos muestran una huella genética de origen norteafricano en las razas bovinas del sur de Europa, basándose en la hipótesis de una migración desde África a través de la cuenca mediterránea durante el Neolítico. Estos alelos de cebú se han observado, igualmente, en Pirenaica y Terreña apoyando de nuevo la migración desde África a la zona mediterránea, pero que también llegó a la región del País vasco durante las migraciones humanas y ganaderas del Neolítico. Las relaciones filogenéticas con otras razas bovinas europeas han mostrado una diferenciación genética bien definida de Salers, que parecía estar genéticamente más próxima a las razas Blonda de Aquitania, Limusina y Charolesa. Por otra parte, la Pirenaica y la Terreña, ambas razas autóctonas del País Vasco, mostraron cierta mezcla genética aunque se separaron a nivel genético correctamente de otras razas alóctonas criadas en la región (Salers y Frisona).

La segunda parte de esta tesis se centró en la caracterización de los AG en ganado vacuno y su relación con los genes lipogénicos: estearoil-CoA desaturasa (isoformas *SCD1 y SCD5*) y la proteína 1 de unión a los elementos reguladores de esteroles (*SREBP1*). Es la primera vez que se relaciona la genética de las razas bovinas criadas en el País Vasco con características de la calidad de la carne, concretamente con el perfil de AG. Según la evidencia científica, aún no se conoce con detalle los

mecanismos y vías bioquímicas por las cuales los genes lipogénicos afectan a la composición de los AG. Por tanto, esta parte de la tesis está enfocada en el análisis y estudio de la expresión génica y la variabilidad de varios genes lipogénicos, así como su asociación con la composición de los AG en tejido subcutáneo de cuatro tipos comerciales criados en el País Vasco (toros de Pirenaica, novillas de Pirenaica, toros de Salers y vacas de Frisona). Para abordar este objetivo, en primer lugar, se ha llevado a cabo una identificación de isómeros de AG utilizando tres *runs* de cromatografía de gases y dos columnas capilares. En segundo lugar, se utilizo transcripción inversa mediante co-amplificación y PCR cuantitativa para analizar la expresión genética de los genes lipogénicos *SCD1*, *SCD5* y *SREBP1*.

En primer lugar, se investigó el perfil de AG y la expresión génica del tejido adiposo en los cuatro tipos comerciales. La composición de AG reveló diferencias significativas en especies específicas de AG saturados, monoinsaturados y poliinsaturados. El efecto de la expresión génica de los genes lipogénicos sobre la desaturación y la composición de los AG parece estar influenciado por la raza y el sexo. Además, la correlación opuesta observada entre las dos isoformas *SCD1* y *SCD5,* sugiere un modelo de compensación génica entre la expresión de ambos genes, que a su vez regula la actividad enzimática de desaturación de los AG.

Segun la literatura científica, varias variantes en la secuencia de los genes lipogénicos han mostrado estar asociadas al perfil de los AG. Sin embargo, se desconoce el mecanismo que subyace a la relación entre estos polimorfismos, la expresión génica y la composición de los AG. En este sentido, el polimorfismo de inserción/deleción de 84 pb (84 pb-indel; rs133958066), ha sido asociado previamente al perfil de AG, aunque no sé conoce su función o posible efecto en la regulación del gen *SREPB1*. Por lo tanto, se ha estudiado este indel, junto con la expresión génica y el perfil de los AG en los principales tipos comerciales descritos anteriormente. Este estudio describe, por primera vez, el alelo S y el genotipo SS del indel de 84 pb en Pirenaica y Salers. Además, estos resultados han mostrado la mayor frecuencia alélica del alelo S, en comparación con otras razas bovinas europeas. El alineamiento de la secuencia nucletídica de *SREBP1* reveló que el intrón 5, que contiene el indel de 84 pb, muestra una mayor similitud entre rumiantes en comparación con otros intrones de *SREBP1*,

lo que podria sugerir que se trata de una región altamente conservada a lo largo de la evolución. Los resultados han demostrado la asociación del polimorfismo 84 bp-indel y la composición de AG en los tipos comerciales estudiados, especialmente en la raza Pirenaica, donde el alelo S y el genotipo SL se asociaron con mayores contenidos de AG saturados (18:0) y poliinsaturados (18:3n-3) de grasa subcutánea. Por tanto, la región de 84 pb-indel parece tener una función por aclarar en el gen *SREBP1* o incluso podría actuar regulando los niveles de transcripción de otros genes lipogénicos.

En conclusión, los resultados obtenidos en la presente Tesis Doctoral confirman que las herramientas genéticas investigadas son útiles para estudiar con precisión el parentesco biológico, la identificación y controlar la trazabilidad en la industria alimentaria. Además, el enfoque genético desarrollado para el estudio de los genes relacionados con la homeostasis y el metabolismo de los lípidos, combinado con el análisis del perfil de AG, contribuye a la comprensión de los caracteres asociados a la calidad de la carne. Este conocimiento es relevante para su uso en nuevas estrategias de selección, reproducción y una gestión sostenible con el objetivo de producir carne y productos derivados de alta calidad, como demandan las recomendaciones sanitarias actuales.

SUMMARY

This Ph. D. Thesis has been conducted in collaboration between BIOMICs and Lactiker Research Groups at the University of the Basque Country (UPV/EHU), which are dedicated to perform multidisciplinary research in the field of genetics, and quality of foods from animal origin, respectively.

The agri-food sector is of great importance to the European economy. Indeed, the European Union (EU) is the largest global exporter of agri-food products, while Spain is the 3rd producer of the EU. Within Spain, in the Autonomous Community of the Basque Country (later on Basque Country), the bovine sector represents the main economical value of livestock production and this is very much linked to the ancient Basque life-style and pastoralism. Regarding the food production, new breeding policies at EU level are marking a trend towards the maintenance of sustainable production systems using native animal genetic resources. To achieve this, the effective management of farm animal genetic resources demand clear knowledge of each breed characteristics, involving geographical distribution, the production environment, within- and between-breed genetic diversity and population data among others. Simultaneously, the genetic selection is an on-growing strategy to improve production as a response to new markets, and becoming the most efficient and practical way to improve meat quality traits. It is well known that every breed has a wide range of variability in its genetics, and the understanding of its relationship with meat quality traits has become a very relevant and necessary step for marker assisted selection..

The main objectives of this Ph. D. Thesis were (1) to perform a genetic characterization of several bovine breeds well established in the Basque region in order to study their genetic diversity, population structure, founder effect, historical migrations alleles, the effectiveness of genetic assessments of different STR marker panels, and phylogenetic relationships with other bovine European

breeds; and (2) to characterize the fatty acid (FA) composition of adipose tissue obtained from different commercial types (obtained from several breeds produced in the Basque region) in order to associate this profile to the gene expression and polymorphisms of several lipogenic genes.

Genetics has become a powerful tool for the consistent characterization of animal genetic resources and a useful approach to study the diversity of animal species and livestock breeds. Likewise, microsatellites (STR) have demonstrated to be effective genetic biomarkers to support animal parentage and identification cases. In order to address the first objective, the analytical methodologies applied moved from conventional PCR to fragment analysis through sequencing by capillary electrophoresis. To start, the efficacy of a commercial 12-STRs panel was evaluated in Terreña and Salers cattle populations for cattle parentage and meat traceability testing. The results showed that Combined Power of Exclusion (CPE) values were directly related to the number of loci analysed, and concluded that this marker set was sufficient for the identification of an individual. However, increasing the number of STR makers to ensure a trustworthy parentage is recommended.

Salers cattle, originally from France, was introduced in the Basque region and expanded to other regions of Spain due to the rapid specialization of livestock management using highly productive allochthonous breeds. In the second study, the previously used 12-STR commercial panel was applied for genetic characterization and the evaluation whether a founder effect have occurred in Spanish Salers due to the low number of initial reproducers. When population was studied using Sign test and Wilcoxon's sign rank test performed under stepwise mutation and two-phase mutation models, the allochthonous Salers revealed the absence of a founder effect in the expansion of this breed. These analyses showed that Salers population seemed to be under mutation-drift equilibrium due to a constant incorporation of breeding animals to the population.

The native Pirenaica cattle is of great importance as local animal genetic resource and obtained beef is registered under a Quality Label included in a Protected Geographic Indication in the Basque region. According to our first results, an increase in the amount of STR markers analysed was recommended and, therefore, as next step, the genetic effectiveness of ISAG/FAO 30-STR panel was investigated for parentage, individual identification and the selection of the most discriminant markers in Pirenaica and other breeds typically reared in the Basque Country (Terreña, Blonde d´Aquitaine, Limousin, Salers and Holstein-Friesian). These results revealed that a minimum of 21-STR panel is necessary for a reliable parentage study in the absence of genetic information from one parent and for trustworthy genetic assignments based in machine-learning predictive models.

In general, the genetic characterization of native Pirenaica, Terreña and allochthonous Salers revealed the presence of African zebu diagnostic alleles (BM2113-123, BM2113-131, BM2113-143 and ETH152-193). In Salers, these alleles supported the northern African genetic signature in southern European cattle breeds based on the hypothesis of dispersal through the Mediterranean basin during the Neolithic period. These zebu alleles were interestingly observed in Pirenaica and Terreña supporting again the migration from Africa to the Mediterranean area, that also reached the Basque region due to Neolithic human and livestock migrations. Phylogenetic relationships with other European cattle breeds revealed a well-defined genetic differentiation of Salers which appeared to be genetically close to Blonde d´Aquitaine, Limousin and Charolais breeds. On the other hand, Pirenaica and Terreña, both native breeds from the Basque region, showed certain admixture although they were genetically separated from other allochthonous breeds raised in the region (Salers and Holstein-Friesian).

The second part of this thesis was focused on the FA characterization of cattle and its relationship with lipogenic *stearoyl-CoA desaturase* (*SCD1* and *SCD5* isoforms) and *sterol regulatory element-binding protein-1* (*SREBP1)* genes. This is the first time where genetics of cattle breeds reared in the Basque region have

been related to meat quality parameters; concretely FA profile. Up to date, the mechanisms by which lipogenic genes affect the FA composition and the related biochemical pathways are not fully understood. Consequently, this part is dedicated to investigate the expression level and variability of several lipogenic genes and their associations with the FA composition of subcutaneous fat from four major commercial types reared in the Basque Country (Pirenaica bulls, Pirenaica heifers, Salers bulls and Holstein-Friesian cull cows). To address this objective, first of all, an adequate FA isomer identification was performed using three GC runs and two capillary columns. Secondly, reverse transcription and quantitative PCR by co-application reverse transcription method was developed to analyse gene expression of *SCD1*, *SCD5* and *SREBP1* lipogenic genes.

Initially, the FA profile and the gene expression of adipose tissue were investigated in the four commercial types. The FA composition of backfat tissue revealed significant differences in specific SFA, MUFA and PUFA species among commercial types. The effect of gene expression of lipogenic genes on the desaturation and FA composition seemed to be influenced by breed and gender. And, the opposite correlation observed between *SCD1* and *SCD5* isoforms suggests a novel genetic compensatory regulation of the SCD activity.

In the scientific literature, several sequence variants in lipogenic genes have shown to be associated to FA profile. However, little is known about the mechanism underlying the relationship between these polymorphisms, gene expression and FA composition in beef. In this sence, the 84 bp insertion/deletion polymorphism (84 bp-indel; rs133958066) has been previously related to FA profile although its function or posible effect in *SREPB1* regulation is not well understood. Therefore, this indel, together with gene expression and FA profile has been studied in the major, previously described, commercial types. In Pirenaica and Salers, the 84 bp-indel presented the S allele and SS genotype which has been reported for the first time. Moreover, the results obtained support their highest S allele frequency compared to other European beef cattle breeds reported in the literature. The alignment of *SREBP1* sequence revealed

that the non-coding intron 5, which contains the 84 bp-indel, showed higher similarity among ruminants compared to other introns of *SREBP1*, suggesting that this could be a well-conserved region throughout evolution. Also, the association of the 84 bp-indel polymorphism and the FA composition of studied bovine commercial types has been demonstrated, especially in Pirenaica breed, where S allele and SL genotype were associated with higher saturated (18:0) and polyunsaturated (18:3n-3) FA contents in subcutaneous fat. Therefore, the 84 bp-indel region may have a function in *SREBP1* or even could act regulating transcription levels of downstream genes.

In conclusion, the results obtained in the present Ph. D. Thesis confirm that genetic tools investigated proved to be useful to accurately assess parentage, identification in cattle and control the traceability needed in the meat industry. Furthermore, the genetic approach developed for the study of lipid homeostasis and metabolism related genes combined with FA profile analyses contribute to the understanding of meat quality traits in beef cattle. This knowledge becomes of relevance for appropriate breeding c and sustainable management practices with the aim to produce high quality meat and derived-products in response to actual health recommendations.

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AKNOWLEDGEMENTS TO THE EDITORIALS

Authors thank the editorials for granting permission to reuse the published articles in this thesis. The published versions can be accessed at the following links:

- Gamarra et al., (2015), *Forensic Science International: Genetics Supplement Series*, 5 253 - 255. (Appendix I) https://doi.org/10.1016/j.fsigss.2015.09.101
- Gamarra et al., (2017) *Animal*, *11*(1), 24–32. (Appendix II) https://doi.org/10.1017/S1751731116001063
- Gamarra et al., (2020) *Animals*, *10*(9), 1584. (Appendix III) https://doi.org/10.3390/ani10091584
- Gamarra et al., (2018) *BMC veterinary research*, *14*(1), 167. (Appendix IV) https://doi.org/10.1186/s12917-018-1481-5
- Gamarra et al., (2021). *Animal science journal*, *92*(1), e13521. (Appendix V) https://doi.org/10.1111/asj.13521

A mis padres y mis abuelos. A Etsuko…

El Templo de la Naturaleza (1802) de Erasmus Darwin (1731-1802)

"LA VIDA ORGÁNICA bajo las olas lejos de las costas,
Nació y creció en las cavernas perladas del océano;
Las primeras formas diminutas, no vistas por lentes esféricas,
Se movían en el lodo, o atravesaban la masa de agua;
A medida que florecen las generaciones sucesivas,
Adquieren nuevas fuerzas y extremidades más largas;
Donde grupos incontables de vegetación surgen,
Y mundos que respiran, de aletas, patas y alas.

Así el alto roble, el gigante del bosque
Que lleva los truenos de Britania en la inundación;
La ballena, monstruo desmedido de la corriente,
El león, monarca de la llanura,
El águila que se eleva en los reinos del aire,
Cuyo ojo no deslumbrado bebe el resplandor solar,
El hombre imperioso, que gobierna la multitud bestial,
De lenguaje, razón y reflexión orgulloso,
Con la frente erguida, que desprecia este césped terrenal,
Y se considera la imagen de su Dios;
Surgió de los rudimentos de la forma y el sentido,
Un punto de embrión, o un ente microscópico.
Conscientes del presente, no ciegos al futuro,

Relacionan el razonamiento del reptil con el de la humanidad.
- Inclínate, orgullo arrogante, observa en conjunto las formas emparentadas,
¡Tu hermana la hormiga, tu hermano el gusano!"

Darwin, Erasmus (1803) The temple of nature: or, The origin of society: a poem, with philosophical notes. J. Johnson.

"ORGANIC LIFE beneath the shoreless waves
Was born and nurs'd in Ocean's pearly caves;
First, forms minute, unseen by spheric glass,
Move on the mud, or pierce the watery mass;
These, as successive generations bloom,
New powers acquire, and larger limbs assume;
Whence countless groups of vegetation spring,
And breathing realms of fin, and feet, and wing.

Thus the tall Oak, the giant of the wood,
Which bears Britannia's thunders on the flood;
The Whale, unmeasured monster of the main,
The lordly Lion, monarch of the plain,
The Eagle soaring in the realms of air,
Whose eye undazzled drinks the solar glare,
Imperious man, who rules the bestial crowd,
Of language, reason, and reflection proud,
With brow erect, who scorns this earthy sod,
And styles himself the image of his God;
Arose from rudiments of form and sense,
An embryon point, or microscopic ens!
Wise to the present, nor to future blind,

They link the reasoning reptile to mankind!
Stoop, selfish Pride! survey thy kindred forms,
Thy brother emmets, and thy sister Worms!"

En primer lugar, me gustaría agradecer a todas las personas que de algún modo me han apoyado en este viaje científico, en el que equivocándome, tropezando he intentado crecer. Ahora siendo sincero e incluso torpe en explicar lo mejor de cada uno, espero al menos engrandecer a cada una de esas personas que estuvieron cerca expresándoles mi más sincera gratitud.

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ABBREVIATIONS

Ag^+-SPE	silver ion – solid phase extraction
AnGR	animal genetics resources (for food and agriculture)
BOE	Boletin Oficial del Estado (Official Newsletter of the Spanish State)
BCFA	branched-chain fatty acid
bp	base pair
c	*cis*
C/F	concentrate to forage ratio
CLA	conjugated linoleic acid
CNV	copy number variation
DAD-IS	Domestic Animal Diversity Information System of the Food and Agriculture Organization of the United Nations
DNA	deoxyribonucleic acid
EAAP	European Federation of Animal Science
EDTA	ethylenediamine tetra-acetic acid
EU	European Union
EUROSTAT	European Union Statistics Office
EUSTAT	Euskal Estatistika Erakundea (Basque Statistics Institute)
FA	fatty acid
FAME	fatty acid methyl ester
FAO	Food and Agriculture Organization of the United Nations
FIS	within-breed fixation index
HW	Hardy-Weinberg equilibrium
IAM	infinite alleles model
INDEL	insertion - deletion polymorphism
ISAG	International Society for Animal Genetics
LD	linkage disequilibrium
MAF	minor allele frequency
MoDAD	measurement of domestic animal diversity
MCMC	Markov Chain Monte Carlo
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial deoxyribonucleic acid
MUFA	monounsaturated fatty acid
NGS	next-generation sequencing
PCR	polymerase chain reaction
PDO	protected designation of origin
PGI	protected geographic indication
PUFA	polyunsaturated fatty acid
QTL	quantitative trait locus
RFLP	restriction fragment length polymorphism
SFA	saturated fatty acid
SER	sterol regulatory element
SMM	stepwise mutation model

SNP	single nucleotide polymorphism
t	*trans*
TPM	two-phase model
UPV/EHU	University of the Basque Country
UN	United Nations
USA	United States of America
VA	vaccenic acid
VNTR	variable number of tandem repeat
WHO	World Health Organization

UNITS

%	percentage	**min**	minute
µm	micrometre	**ml**	mililitre
µl	microliter	**mm**	milimetre
bp	base pair	**ng**	nanogram
g	acceleration due to gravity	**nm**	nanometre
g	gram	**M**	molar
h	hour	**rpm**	revolutions per minute
kg	kilogram	**s**	second
L, l	litre	**°C**	degree centrigade
m	metre	**v**	volume
mg	miligram	**w**	weight

GENE AND ENZYME NOMENCLATURE

ACAC	acetyl-CoA carboxylase
ACL	ATP-citrate lyase
C/EBPα	CCAAT/enhancer binding protein alpha
ELOVL	fatty acid elongase
EGR2	early growth response protein 2
FADS	fatty acid desaturase
FASN	fatty acid synthase
INSIG	insulin-induced protein
LXR	liver X receptor
NF-1	nuclear factor-1
NF-Y	nuclear factor-Y
PPARα	peroxisome proliferator-activated receptor alpha
SCD	stearoyl-CoA desaturase
Sp1	Sp1 transcriptional factor
SCAP	cleavage of activation by SREBP protein
SREBP	sterol regulatory element binding protein

FATTY ACID NOMENCLATURE

	Ab	Systematic Name	Common Name
10:0		decanoic acid	caprid acid
12:0		dodecanoic acid	lauric acid
13:0		tridecanoic acid	tridecylic acid
14:0		tretradecanoic acid	myristic acid
15:0		pentadecanoic acid	pentadecylic acid
16:0	PA	hexadecanoic acid	palmitic acid
17:0		heptadecanoic acid	margaric acid
18:0	SA	octadecanoic acid	stearic acid
19:0		nonadecanoic acid	nonadecylic acid
20:0		eicosanoic acid	arachidic acid
22:0		docosanoic acid	behernic acid
24:0		tetracosanoic acid	lignoceric acid
9*c*-14:1		tetradecenoic acid	myristoleic acid
9*c*-15:1		9*c*-pentadecenoic acid	-
9*c*-16:1		9*c*-hexadecenoic acid	palmitoleic acid
9*c*-17:1		9*c*-heptadecenoic acid	-
9*c*-18:1	OA	9*c*-octadecenoic acid	oleic acid
11*t*-18:1	VA	11*t*-octadecenoic acid	vaccenic acid
9*c*-19:1		9*c*-nonadecenoic acid	-
9*c*-20:1		11*c*-eicosenoic acid	gadoleic acid
9*c*-22:1		9*c*-docosenoic acid	erucic acid
9*c*-24:1		15*c*-tetracosenoic acid	nervonic acid
9*c*,11*t*-18:2	RA	9*c*,11*t*-octadecadienoic acid	rumenic acid
18:2n6	LA	9*c*,12*c*-octadecadienoic acid	linoleic acid
18:3n3	ALA	9*c*,12*c*,15*c*-octadecatrienoic acid	α-linolenic acid
18:3n6	GLA	6*c*,9*c*,12*c*-octadecatrienoic acid	γ-linolenic acid
20:4n6	AA	5*c*,8*c*,11*c*,14*c*-eicosadienoic acid	arachidonic acid
20:5n-3	EPA	5*c*,8*c*,11*c*,14*c*,17*c*-eicosapentaenoic acid	clupanodonic acid
22:6n-3	DHA	4*c*,7*c*,10*c*,13*c*,16*c*,19*c*-docosahexaenoic acid	cervonic acid

Ab: Abbreviation

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SECTION I.

I. STATE OF THE ART

1. GENERAL FRAMEWORK

1.1. THE AGRI-FOOD SECTOR

The agri-food sector is of great importance to the European economy being one of the most prominent industrial sector in the European Union (17 % of total industrial production). Moreover, European Union (EU) is the largest global exporter of agri-food products, being Spain the 3rd producer (EUROSTAT, 2020). The economic importance and the ubiquity of food in our lives determine that food quality and traceability must be one of the main interests of society, and especially, of public authorities and producers in the sector. After the food crises in late 1990s and early 2000s, the Parliament and the European Council, in the framework of the White Paper on Food Safety, adopted a new regulation for the management of all products of animal origin in order to regain consumer confidence and ensure adequate standards of food safety (European commission, 2020a). In fact, the EU regulation establishes a system for the identification and registration of bovine animals and regarding the labelling of beef and derived products (European Parliament, 2000).

On the other hand, the "World Watch List for Domestic Animal Diversity" reported more than 6300 breeds of livestock belonging to 30 domesticated species (FAO, 2003), together with a large proportion of indigenous livestock population of developing countries that might not be considered yet. These great diversity on local populations has been the result of several evolutionary forces such as mutation, environmental adaptation, isolation and genetic drift (Groeneveld et al., 2010), but also the domestication, natural and human selection over the past 12,000 years, leading to the formation of numerous well-defined livestock breeds. It is considered that the breeding programmes carried out during the last 40 years in most of the countries has dedicated their efforts towards more efficient strategies accelerating genetic improvement and focussing in a few number of cattle breeds. Therefore, the resulting European cattle populations have become a mixed population of purebred, crossbred and upgraded cattle with variable genetic make up.

More recently, the progress in feeding strategies and related technology has permitted optimal nutrition, while improved traceability systems have led to uniform and strictly controlled production environments. In general, Western countries have been focused on productivity, and therefore, intensification practices that had a huge impact on native and local breeds, taking them, in several cases, into an endangered or extinct situation.

As a result, this progress in the breeding strategies has led to dramatic changes in livestock biodiversity, increasing the concern about the damage of genetic resources (FAO, 2007). Instead, less productive breeds may contribute to current or future traits of interest (Notter, 1999; Bruford et al., 2003; Toro et al., 2008) and are considered fundamental for maintaining future breeding options.

At present, new production and breeding policies at EU level are marking a trend to change this situation towards the maintenance of sustainable production systems using native animal genetic resources. These strategies has been detailed in the European Research Area on Sustainable Animal Production as well as in the H2020 – Work Program 2018-2020: Food security, sustainable agriculture and forestry, marine and maritime and inland water research and bioeconomy (European Commission, 2020b). In order to achieve this, the effective management of farm animal genetic resources demand clear knowledge of each breed characteristics, involving geographical distribution, the production environment, within- and between-breed genetic diversity and population data among others.

1.2. LIVESTOCK

1.2.1. HERITAGE

During human migrations throughout Europe, livestock species have been imported and were hybridized with the native wild individuals that inhabited each area, giving rise to a multitude of new breeds (Diamond, 2002, Götherström et al., 2005). This process helped to increase cattle populations size, but also produced individuals better adapted to the environment taking characteristics of the wild species. This process was different along Europe and depending on the local crossbreeding wild animals, a high diversity of breeds with characteristic phenotypes were obtained (Ajmone-Marsan et al., 2010). Many of these breeds living in the same region for centuries have formed the actual native breeds due to environmental adaptation acquired over the centuries and the limited mobility of humans, apart of cattle traders and transhumant herds (Fernandez, 2002).

In the 19th century, compared with other European countries, livestock in Spain evolved differently considering that the industrialisation process started later and was slower due to the Civil War, that did not favour the process of agricultural modernisation

(Naredo, 2004). Therefore, the traditional agricultural model was maintained until the 1950s, where the Spanish livestock agro-systems were limited to the pasture availability, while native breeds represented 74 % of cattle livestock (MAPA, 1986). In the following 45 years, however, a rapid transformation happened towards a more industrialized livestock production, although small cattle farms were dominant (83 %) during 2005 (Ríos-Núñez et al., 2013). Over the last decade, the number of registered farms has decreased, even this trend is not accompanied by a fall in the number of animals, indicative of a reconversion and productive specialisation (intensification) of farms (MAPA, 2020).

The Autonomous Community of the Basque Country (later on Basque Country), located at the Cantabric Sea and the western end of the Pyrenees, is one of the 17 regions that make up the Spanish state. There is an estimated population over 2.19 million for a total surface of 7,261 Km, which means a high population density (303 inhabitants/Km) (EUSTAT, 2021), mainly located in the coastline and in the valleys due to an abrupt and mountainous orography. The Basque livestock production has a spatial and economic significance in this region; the climatic and topographic characteristics of the region have supported its development. The ancient Basque life-style linked to pastoralism is well known and has played an important role in the management of local lands (communal, faceries). Indeed, many of these traditional grazing areas are currently within the Natura 2000 Network (Natural Parks, Sites of Community Interest, Special Area of Conservation, etc.) that covers over 20 % of the Basque geography (European Environmental Agency, 2019). Likewise, the contribution of livestock activity to the Basque society cannot be evaluated solely in economic terms and over the centuries has left obvious traces in the cultural heritage (i.e., *idi-probak* - oxen competitions in Basque language), and a significant number of small-sized producers are committed to manage the territory through agricultural and extensive livestock activity in family-owned farms (5 to 30 ha) called *baserri* which was subsisted majorly by a process of *mayorazgo* (primogeniture).

Basque Government assumed competences in Agriculture and Livestock in 1981, when the local primary sector consisted of a huge number of these small-sized non-specialized family farms. Then, the imminent Spanish entry in the EU offered an uncertain future for many farms due to the fast development of industry, which made more difficult the consolidation of a solid agriculture (Amenabar et al., 2006). However, the sector was considered of strategic importance and it begun a process of structural organization (Urarte et al., 1999), which later on adopted effective regulation measures in terms of

health and quality of derived products towards a more competitive and sustainable livestock production.

1.2.2. CONSERVATION OF ANIMAL GENETIC RESOURCES

The need of characterization and conservation of animal genetic resources (AnGR) has been promoted as a main priority at national and international level (United Nations, 1992). In addition, this conservation should be connected to a sustainable development considering a rational use of the animal genetic resources adequate to their environmental surroundings. Currently, the conservation and sustainable use of AnGR is considered an activity of public benefit (Hodges, 2002).

The AnGR has been classified according to the threat status. However, there is no common criteria to establish the risk status of a population although there are many factors that can influence the definition of risk status (health, social, political, economic, cultural or genetic). The EU determined that a cattle population bellow 7500 animals could be considered "in danger of being lost to farming" (Commission Regulation (EC) No 445/2002). However, the analysis of the Global Databank for Farm Animal Genetic Resources (FAO, 1998), classified breeds' risk status as reported in **Table 1.1.**, based on overall population size, number of breeding females, number of breeding males, the percentage of females bred to males within the same breed, and the trend of population size. Further consideration is given to whether active conservation programmes are in place, or not, for critical or endangered populations. When relevant information on conservation management of breeds at risk is not available a conservative approach is taken and the breed is categorised at the higher risk category of critical or endangered.

Table I.1. Classification for animal breeds based on seven categories for animal breeds (FAO, 1998).

	BREEDING FEMALES (n)	BREEDING MALES (n)	OVERALL POPULATION (n)	FEMALE / MALE RATIO
EXTINCT	0			
CRITICAL	≤ 100	≤ 5	≤ 120 individuals and decreasing	< 80 %
CRITICAL-MAINTAINED	Critical breed status for which active conservation programmes are in place or populations are maintained by commercial companies or research institutions.			
ENDANGERED	100 - 1000	5 - 20	80 - 100 and increasing	> 80 %
ENDANGERED-MAINTAINED	Endangered breed status for which active conservation programmes are in place or populations are maintained by commercial companies or research institutions.			
NOT AT RISK	> 1000	> 20	> 1200	> 80 %
UNKNOWN	There is no information available to assess the situation of the population.			

According to the Second Report on the State of the World's AnGR for Food and Agriculture, 13 % of world's livestock breeds are already extinct, while the breeds classified as being at risk of extinction increased from 15 % to 17 % between 2005 and 2014 (FAO, 2015). Moreover, several regions have much higher proportion of their breeds classified at risk such as Europe and the Caucasus (54 %; DAIS-IS, 2021). In fact, 13 % of the cattle breeds are endangered or in critical situation in the world (**Figure 1.1.**).

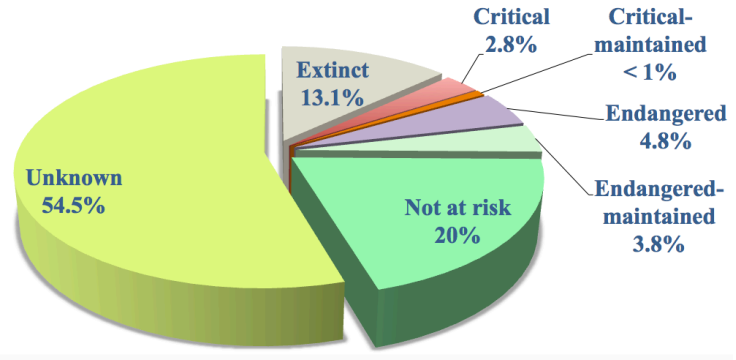


Figure I.1. Worldwide cattle breeds classified by risk status category in percentage basis.

As aforementioned, during last decades, the specialization of animal production has been one of the greatest threats for animal genetic diversity. In developed countries, commercial cattle livestock production has been based on the management of a very few highly selected breeds focused into the large production, reducing variability within highly productive breeds and promoting the progressive replacement of local breeds by more specialized ones. However, many reasons sustain the need for the conservation of Genetic diversity of AnGR (Oldenbroek, 1999). Therefore, conservation become one of the four Strategic Priority Areas of the adopted Global Plan of Action for AnGR (FAO, 2007), underlining the need for national governments to address this topic. Genetic diversity permits the capability of a breed or population to respond to selection for adaptation to changing environmental conditions, considering those conditions associated with climate, but also changes in markets, management and farming practices or disease risks. In addition, diversity of AnGR also helps to preserve cultural and historical values in develop countries, to sustain the inheritance value of local livestock, which is linked to the emergence of niche markets for livestock products, while food security and economic development are the immediate concerns in developing countries.

In Spain, autochthonous breeds constitute the rich heritage of spanish livestock farming and are, moreover, singular within the context of livestock in the European Union. The systems of management used are related to the geographical characteristics of the different territories in which they are risen. According to the Official Catalogue of Breeds of Spain, there are 165 autochthonous livestock breeds, from which 140 are endangered. In cattle, the situation is also delicate considering that from 38 autochthonous cattle breeds, 31 are endangered (MAPA, 2022).

1.2.3. ECONOMIC VALUE OF LIVESTOCK ACTIVITY

In Spain, beef livestock activity is a strategic sector of economic significance and it represents 5.8 % (3.092,6 million €) of the total agricultural production. It increased a 36 % in the last 10 years becoming the 3rd beef producer, after France and Germany, with 8.68 % of the total registered cattle heads at EU level (FAOSTAT, 2020). Beef cattle represents the 4th livestock production sector in Spain (15.2 %) (**Figure I.2.**) (MAPA, 2021). Taking into consideration the type of bovines slaughtered; 34 % are of 8-12 months of age, 31 % are males over one year old, 19 % are heifers, 13 % are cows and 3 % are calves, while the slaughter of calves under 8 months is low in Spain (3 % of total calves slaughtered). This contrasts with other European countries like the Netherlands

or France, where the number of calves under 8 months slaughtered is significantly higher. Also, it is worth mentioning that over the years the consumer demand of meat protected under quality and/or organic certification labels has increased.

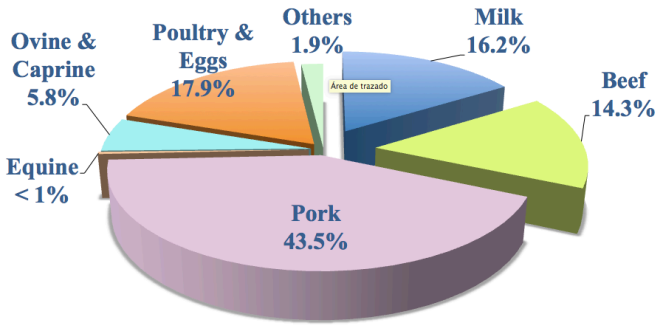


Figure I.2. Economical value of livestock production in Spain (20,178 million €, MAPA, 2021).

Considering the distribution of beef livestock across Spain in 2020, Catalonia concentrates 18 % of the national beef production and Castilla y León the 17 %, which together with Galicia and the Valencian Community, account for 60 % of Spanish beef production.

In the Basque Country, and according to the latest published data and statistics of 2016, livestock production has been estimated at 175 million €, which currently represents around 32 % (543 million €) of final agricultural production. In the Basque Country, in contrast with other Spanish regions the bovine sector represents the main livestock production with the 80.5 % of the economical value (**Figure I.2.** & **Figure I.3.**) from which 69 % of the farms are dedicated to beef and 31 % to dairy production (EUSTAT, 2016).

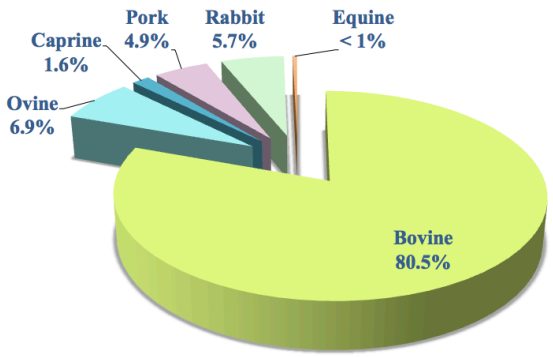


Figure I.3. Economical value of meat production in the Basque Country.

1.2.4. CATTLE BREEDS

In the Basque Country, purebred Pirenaica, Terreña, Monchina and Betizu cattle breeds are conserved as local genetic resources, and several of them (Pirenaica, Terreña and Betizu) are registered as a "100 % autochthonous breed" by the Spanish Ministry of Agriculture under the National Regulation (R.D 2129/2008, December 26th) on conservation, improvement and promotion of animal breeds (BOE, 2008).

Pirenaica remains as the most important breed in terms of numbers, years of experience in conservation and genetic improvement. In fact, Pirenaica is the only autochthonous beef breed raised in commercial flocks with productive purposes and has a genetic programme established by the Basque Country and Navarre regions. Moreover, the traditional management of local breeds in Basque Country has led to high quality foods such as beef with Basque Quality Label included in a Protected Geographic Indication (PGI). This PGI classifies mainly Pirenaica, other breeds and its cross-breeds in heifer/bull calf (8-20months), adult animal (21-84 months) and steers (21-59 months) that are borned, reared and slautghtered in the Basque Country. Registered animals are reared along traditional lines, as regards the conditions determining their health, welfare and taking special attention to diet, which is based on forage resources (fresh grass, dried grass, hay and straw) and, when necessary, authorised feedingstuffs. (European Commission, 2003).

The southern Basque region (*Hegoalde*) shares a long border with the northern Basque region (*Iparralde* or *Pays Basque Français* in French), across the *Le département des Pyrénées-Atlantiques* of France. The history between these two regions is related to the language *euskera* and common cultural traditions that have historically promoted the commerce between them. This implied the introduction of allothchonous breeds such as the french

Limousin or Blonde d´Aquitaine, which are also recognized within the Basque PGI beef Quality Label. In addittion, other beef or dual purpose cattle breeds have been imported from France such as Charolais or Salers introduced more recently (20th century).

On the other hand, dairy production plays an important role in the agricultural sector of the Basque Country as well as in other Cantabric regions (Cantabria, Asturias, and Galicia). In the Basque region, dairy cattle is not only important because of the number of dairy farms, but also because the cultural values of the *baserri* (family own farm-houses), where the milk production represents the cohesion with the rural territory. This sector had a progressive change since the late 80s, and 50 % of the producers disappeared while more productive farms were established. The European line of Holstein-Friesian was firstly introduced as a dairy production breed well adapted to grazing. Later, the industry introduced genetics from Canada and other countries (highly productive lines). The result was a Holstein-Friesian cow sensitive to extensive management, especially to climate and feed quality changes. In some cases, farms introduced Fleckvieh and Brown Swiss breeds which are better adapted to extensive management and a dual production (meat and milk).

Nowadays the majority of farms (90 %) produce under intensive and in few cases semi-extensive models. Productive animals are stabled whereas heifers, dry cows and males are kept grazing. In general, heifers are moved to milk production, while males and cull cows become part of the local meat production.

1.2.4.1. Terreña

Literally Terreña means "from the land" in spanish, emphasizing its local and rustic characters. Historically, this breed has been very popular in the Basque region due to its dual purpose, work and meat production. In 1916, there were 13,346 working oxen registered in Álava, but its population was dramatically decreased since then, registering about 450 Terreña cows and only 4 active sires in 1999 (Lauzurica et al., 1999). Since 2000, a great effort has been made to recover Terreña population but it is still catalogued as an autochthonous endangered breed, with 75 % of the animals located within a radius of less than 50 km, according to the official census of the Ministry of Agriculture, Fisheries and Food (MAPA, 2022; **Figure I.4.**). Currently, Terreña breed is in expansion with an increase in both, breeding females and livestock farms, thanks to regulatory norms, such as the specific regulation of Terreña cattle breed through the Decree 373/2001 about Basque autochthonous animal breeds and entities dedicated to

their promotion (BOPV, 2003).

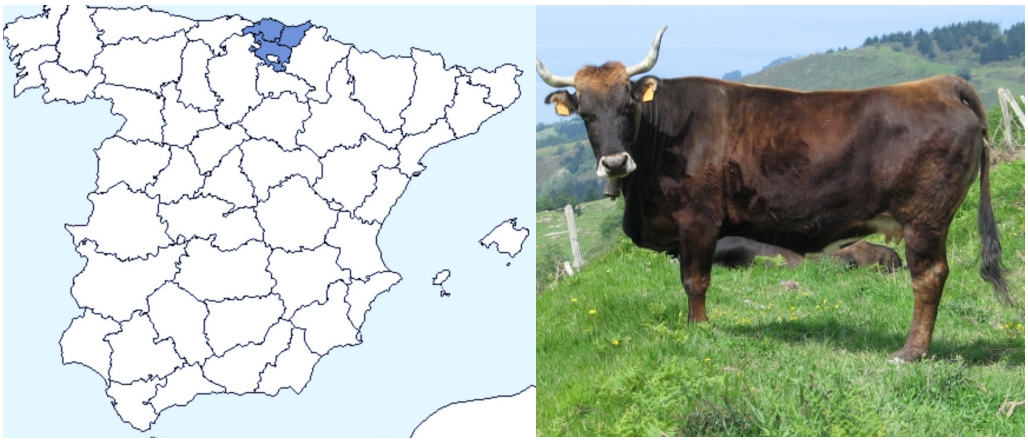


Figure I.4. Left: Distribution of Terreña breed in Spain (MAPA, 2022). Right: An individual of Terreña cattle (Euskal Abereak).

Terreña breed presents a chestnut coat with little muscle mass, good bone mass characterized by a straight forehead and it belongs to the Cantabrian Trunk characterized by brown coats, black mucous membranes and subconcave profile (Lauzurica, 1999). They have thin, white horns with black tips and the females have cream-colored udders (Gomez & Amezaga, 2003). It is characterized by its hardiness and lively temperament typical of mountain breeds. Traditionally, oxen have been used mainly for agricultural work and traditional Basque sports such as "*idi-probak*" or stone dragging.

1.2.4.2. Pirenaica

This breed owes its name to its origin, the Pyrenees, mountainous range located between Spain and France. Pirenaica breed is an autochthonous breed of the Basque Country and Navarra. According to Mendizabal et al. (1998), the Pirenaica population increased significantly during the 1850s and its population was around 80,000 animals during 1857, after the support of breeders and Provincial Councils in the middle of 19th century, but the later introgression of new cattle breeds from Europe lead to an endangered situation of the former breed. Several factors (industrialization and massive migration of rural population to the cities, pasture transformation on crop lands and introduction of more productive breeds) led Pirenaica breed in a serious danger of disappearing, with its highest regression (1500 individuals) in the 1970s (**Figure I.5.**). It was not until 1975, when the need to maintain sustainable production systems using native animal genetic resources promoted the recovery and improvement of Pirenaica. The efforts of breeders

and administrations helped on its recovery due to the promotion and improvement plans promoted by breeders of several spanish regions such as the Basque Country, Navarra, Cantabria, Aragon and Catalonia. The Pirenaica breed herd-book was created in 1905, being the first herd-book in Spain, showing its importance as a breed rooted in these lands and its culture. Even though, it is in a good situation today ("not at risk", **Table I.1.**), this breed still needs the support as every native breed and every genetic heritage deserves. At present, Pirenaica has the largest population size compared to other aforementioned native Basque breeds and it is the first native cattle breed being included in a selection program in the Basque Country. Therefore, the selection from a reduced number of reproducers might imply lower heterozygosity and a reduction of the number of alleles in comparison with other native breeds that have been kept in a semi wild natural environment.

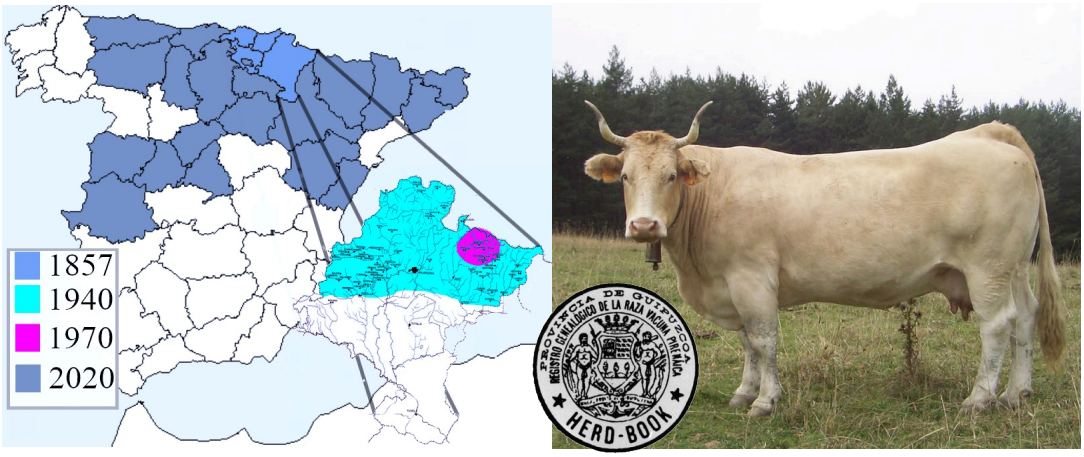


Figure I.5. Left: Distribution of Pirenaica individuals in Spain during its expansion until 1857 (Basque Country and Navarra), its regression from 1940 to 1970 (Navarra), and the most recent recovery until 2020. Right: An individual of Pirenaica breed (local breeder), and the stamp of the first Pirenaica herd-book (1905) from a provincial government (Guipuzkoa) of the Basque Country (Mendizabal et al. 2005).

They are strong mountain animals adapted to harsh Pyrenees climate but also to the mild weather of the Cantabrian coast. They are medium sized animals, with great muscle mass (average carcass weights of 300 kg). They have a single-colored coat, which can be "*gorri*" (red) or "*zuri*" (white). They have pearly white horns facing forward, with yellowish tips. It is worth noting its docility, ease of calving and good suckling ability of cows.

1.2.4.3. Salers

Salers is a breed from the Auvergne region (France) located in the mountainous area called the Massif Central. This region has poor soil and a difficult climate with altitudes of 750 to 2100 m over the sea level. Its ability to handle low temperatures combined with exceptional calving ease explains its popularity, not only across France but also in other regions of Europe, USA, Canada or Australia.

After being introduced in Spain for meat production, it was firstly introduced in the Basque Country in 1985. Afterwards, more animals have been imported making up a Spanish population of 22,000 individuals by 2016, when the Spanish Salers herdbook was created by the ProSalers Association. Since then, Salers population has increased until 90,000 individuals (personal communication).

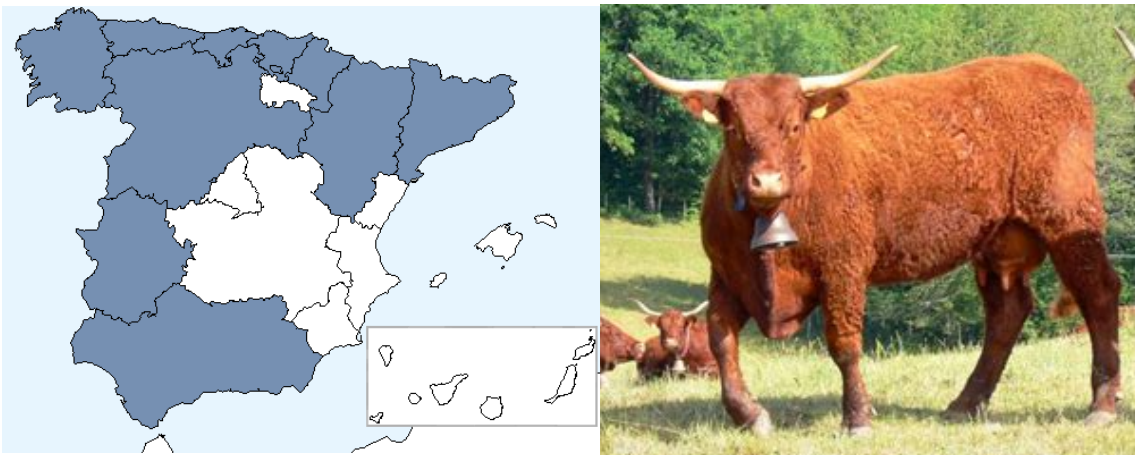


Figure I.6. Left: Distribution of Salers individuals in Spain (ProSalers Association). Right: An individual of Salers breed.

Salers is a rustic dual purpose (milk/meat) breed with a French protected designation of origin (PDO) cheese production. They have a characteristic red colour, and medium to long curly hair, but it is also possible to find black animals. The horns are brownish and elliptical, and the neck is short in bulls, while long and slender in cows. The body is large with a deep chest and the hindquarters run down to the beginning of the tail; well provided with musculature.

1.2.4.4. Holstein-Friesian

Holstein-Friesian is the most widespread cattle breed in the world, being developed by selective breeding programmes over the last 100 years (dairy purpose). Holstein-Friesian was originally from the Netherlands, and later was exported to the United States of America (USA) and improved for milk production. This resulted in large milk producer animals. They are recognized by their distinctive colour patterns of black and white or red and white.

First Friesian heifers were introduced in Spain from Netherlands in the 1950s, as a result of the high milk demand of the growing Spanish population. In the 60s, during the 1st and 2nd Development Plans of the Spanish Government and the need for a highly productive dairy breed, over 15,000 Holstein heifers were imported from USA and Canada, whereas another 3,000 animals were imported from Netherlands, Germany, Great Britain and Sweden as a demand of a European Friesian pedigree by the Spanish breeders (Sanchez, 2002). Since 1990, new embryo transfer programmes replaced animal imports. At present, Holstein-Friesian represents 40 % of the total cattle population in Spain. It is considered as an "integrated" breed, a foreign breed which had genealogy and performance controls, a sufficient census to carry out a breeding program, and a well demonstrated adaptation to the environmental conditions and local production systems (BOE, 2019). Its population is highly concentrated (60 %) in small and extensive/semi-extensive farms in the regions close to the Cantabric Coast of northern Spain (**Figure I.7.**) (MAPA, 2022). In other regions (mainly Mediterranean), bigger intensive farms are located. According to the Agriculture Ministry, Holstein-Friesian population in the Basque Country is distributed in 282 farms and composed by 26,000 individuals used for dairy production (MAPA, 2022). However, there is a current trend for intensification in the Basque Country, where dairy cattle population has decreased, while milk quota in the territory has remained constant (Arriaga et al., 2009). At present, in the Basque region, after their dairy production life cycle, Hostein-Friesian cull cows are kept under grazing conditions while some of them are finished on forages and/or concentrates to improve their fatness before slaughter.

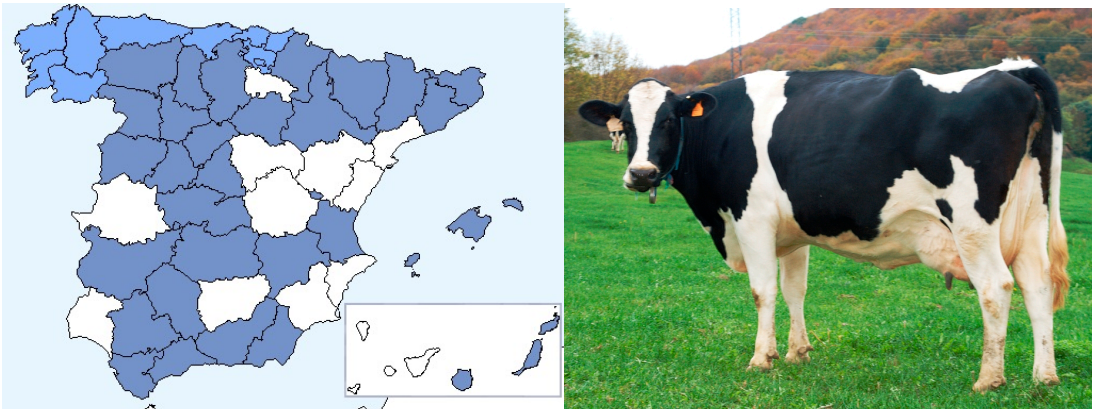


Figure I.7. Left: Distribution of Holstein-Friesian in Spain (MAPA, 2022). Light blue: Regions close to the Cantabric coast; Dark blue: Regions with mainly Mediterranean climate and Canary islands (Box: subtropical). Right: An individual of Holstein-Friesian cattle.

2. LIVESTOCK GENETICS

2.1. GENETIC CHARACTERIZATION

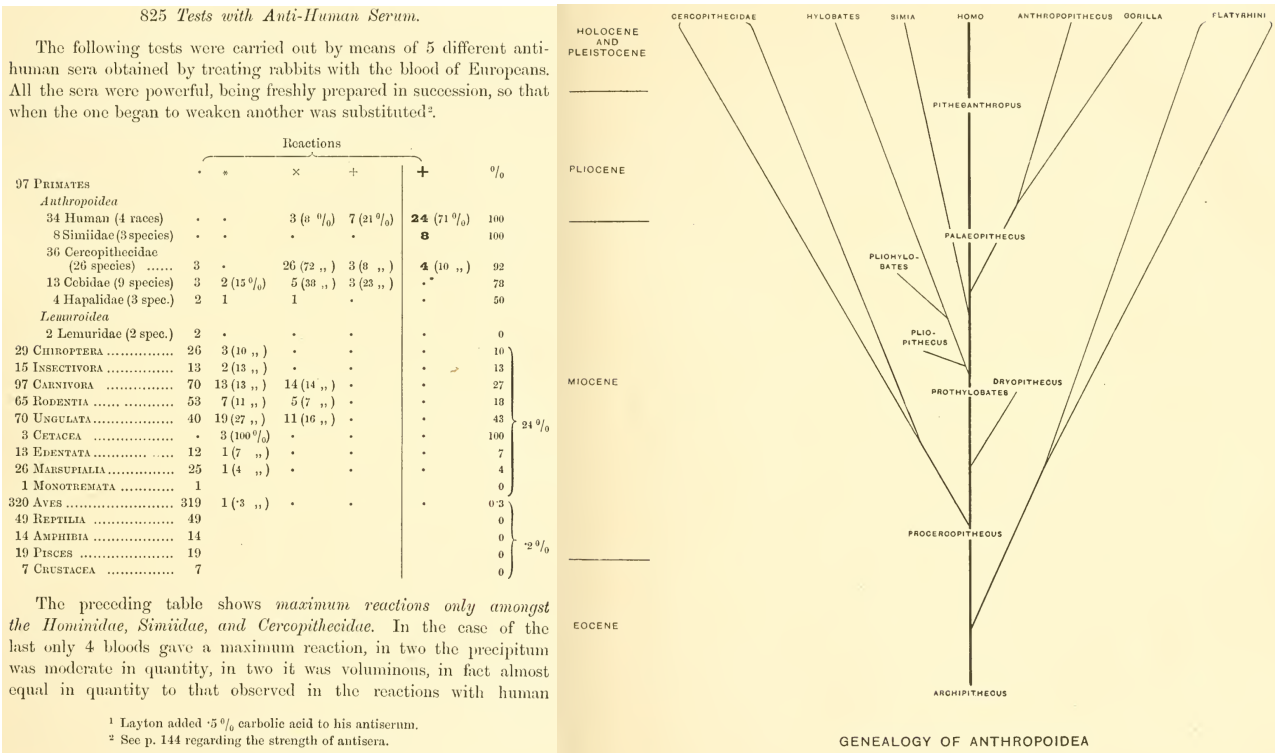
Animal genetics is one of the pillars of livestock development, ranging from characterization to conservation and genetic improvement, which involves actions at local, national, regional and global scales. Genetic characterization assumes the assessment of livestock populations to establish their current status and identify populations' strengths and weaknesses. It helps to the conservation of AnGR allowing the prevention of the loss of genetic diversity in livestock populations, including the protection of the breeds from extinction. Animals are a function of their genes and the environmental influence on them, from whom products (i.e., meat, milk, wool, etc.) and services (i.e., transport, draught power, cultural services, etc.) are determined by the animal breeding strategies (genetic improvement). Therefore, improvement can be achieved by selecting genetically more adapted animals, in terms of a particular set of characteristics, such as productivity, fertility, disease resistance or longevity related to costs of production, but also nutrition value or quality of their products as a demand from the consumer.

Since the middle of the 20th century, genetic improvement efforts have concentrated on a very small number of breeds worldwide. However, the characterization of local breeds is becoming a first step into a future genetic improvement in indigenous breeds, in which local production systems and how they affect animals' ability to survive, produce and reproduce should also be considered. During last decades, genetic characterization

has been accomplished through the study of population genetic diversity parameters and using molecular genetic information. After genetic characterization, new strategies such as cryo-conservation or artificial reproduction are becoming interesting options as second-step approaches for protecting threatened breeds from extinction.

2.1.1. GENETIC POLYMORPHISMS

Assays based on the molecular analysis of genetic variation have been demonstrated as useful tools to study the genetic structure and evolutionary history of biological organisms. The first evidences of genetic variation at the biochemical level were depicted in the past century by Landsteiner (1901) showing heritable variation at the ABO blood group system in humans. Later, Nutall (1904) reported 16000 immunological tests among 900 specimens to deduce relationships among species (Primates, Insectivora, Carnivora, Ungulata, Cetacea, Marsupialia and Aves) trying to place humans in their correct evolutionary position relative to other primates (**Figure I.8.**). In addittion, his results were suggested to be applicable in legal medicine and forensic cases too. However, it was not until the 1950's that the study of definitive structure of the DNA was proposed (Watson & Crick. 1953; Wilkins et al., 1953). And in the 1960s, molecular biology was revolutionised by the study of protein electrophoresis followed by inmunological stains, which revealed that animal genomes harboured an unsuspected wealth of genetic variation (Lewontin & Hubby, 1966).



825 *Tests with Anti-Human Serum.*

The following tests were carried out by means of 5 different anti-human sera obtained by treating rabbits with the blood of Europeans. All the sera were powerful, being freshly prepared in succession, so that when the one began to weaken another was substituted[2].

	Reactions					
	·	*	×	+	+	%
97 PRIMATES						
Anthropoidea						
34 Human (4 races)	·	·	3 (8 %)	7 (21 %)	24 (71 %)	100
8 Simiidae (3 species)	·	·	·	·	8	100
36 Cercopithecidae (26 species)	3	·	26 (72 ,,)	3 (8 ,,)	4 (10 ,,)	92
13 Cebidae (9 species)	3	2 (15 %)	5 (38 ,,)	3 (23 ,,)	·	78
4 Hapalidae (3 spec.)	2	1	1	·	·	50
Lemuroidea						
2 Lemuridae (2 spec.)	2	·	·	·	·	0
29 CHIROPTERA	26	3 (10 ,,)	·	·	·	10
15 INSECTIVORA	13	2 (13 ,,)	·	·	·	13
97 CARNIVORA	70	13 (13 ,,)	14 (14 ,,)	·	·	27
65 RODENTIA	53	7 (11 ,,)	5 (7 ,,)	·	·	18
70 UNGULATA	40	19 (27 ,,)	11 (16 ,,)	·	·	43
3 CETACEA	·	3 (100 %)	·	·	·	100
13 EDENTATA	12	1 (7 ,,)	·	·	·	7
26 MARSUPIALIA	25	1 (4 ,,)	·	·	·	4
1 MONOTREMATA	1					0
320 AVES	319	1 (·3 ,,)	·	·	·	0·3
49 REPTILIA	49					0
14 AMPHIBIA	14					0
19 PISCES	19					0
7 CRUSTACEA	7					0

24 %

·2 %

The preceding table shows *maximum reactions only amongst the Hominidae, Simiidae, and Cercopithecidae.* In the case of the last only 4 bloods gave a maximum reaction, in two the precipitum was moderate in quantity, in two it was voluminous, in fact almost equal in quantity to that observed in the reactions with human

[1] Layton added ·5 % carbolic acid to his antiserum.
[2] See p. 144 regarding the strength of antisera.

Figure I.8. Original immunological test table and animal genealogy tree of George Nutall (1904).

Later, the discovery of bacterial restriction endonucleases that cleave duplex DNA at particular oligonucleotide sequences (Meselson & Yuan, 1968), and the characterization of hundreds of them, proved to be a very useful tool for molecular evolution and population genetics. These enzymes showed to be appropriate to visualize the polymorphisms called restriction fragment length polymorphisms (RFLPs) (Botstein et al., 1980), visualised as changes in the cleavage patterns of DNA fragments. However, the cost in resources and time required to generate the data was huge in comparison with the amount of information obtained. Lastly, the invention of the polymerase chain reaction (PCR) (Mullis et al., 1986), dramatically changed the situation and facilitated economically affordable determination of variants in the sequence of DNA of a large number of individual organisms.

A new era in population genetics started during the decade of 1980, when the analysis of the first highly polymorphic locus called minisatellite or variable number of tandem repeat (VNTR) was reported (Jefreys et al., 1985). The minisatellite showed a high potential for forensic applications due to their high level of variability (Jefreys et al., 1985b), and a new technique for their analysis was developed showing the well-known multiband patterns known as DNA fingerprints (**Figure I.9.**). These molecular markers

revolutionized the population genetics and forensic science, and became the norm for the determination of relationships for both humans and animals.

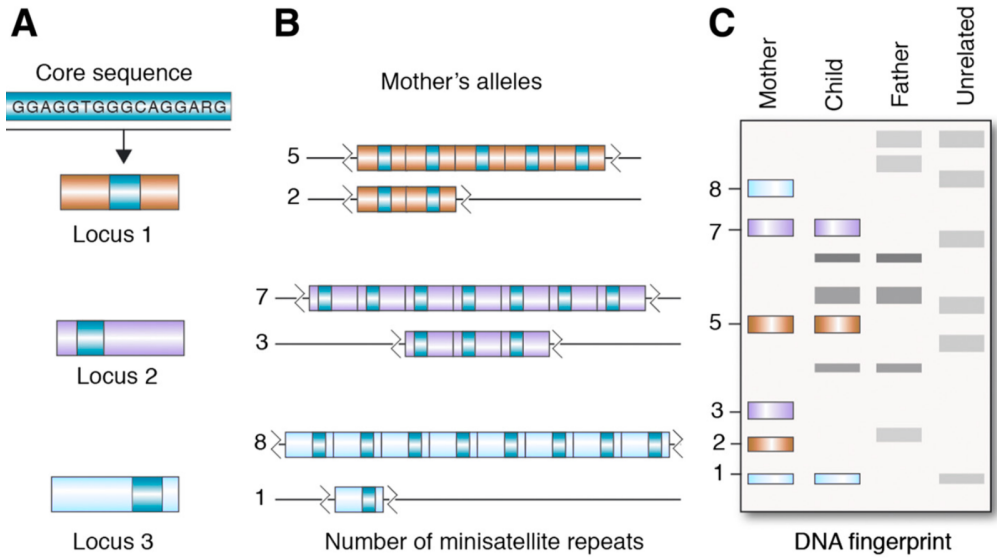


Figure I.9. Minisatellite 16 bp core sequence. (Minisatellite alleles are formed by hundred and thousand of repetitions, this figure shows a simplified representation with few repetitions) (A) A core minisatellite repeat is present at three loci. (B) The number of minisatellite repeats at these loci are shown for one individual (the dam) who is heterozygous at each of the three loci. Locus 1 genotype: 5, 2; locus 2 genotype: 7, 3; and locus 3 genotype: 8, 1. (C) Representation of an autoradiograph showing restriction fragment profiles of four individuals at these three loci. At each locus in the calve's profile, one allele is shared with the dam and the other is shared with the sire, as would be expected when maternity and paternity have been correctly identified. Note that the unrelated individual shares only a small number of bands with the individuals from this family. Modified from Chambers et al., 2014.

Afterwards, the efforts of the geneticist community were directed to the amplification of fragment length polymorphisms, among which microsatellites or short tandem repeats (STRs) are included. Moreover, STRs have become some of the most popular molecular markers in the last 40 years according to the number of citations (**Figure I.10.**; Grover & Sharma, 2014).

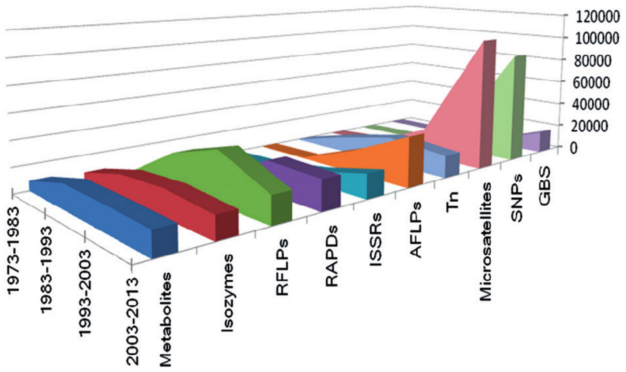


Figure I.10. Popularity of different molecular marker systems in last 40 years. Extracted from Grover & Sharma, 2014. RFLPs: restriction fragment length polymorphisms. RAPDs: random amplified polymorphic DNA. ISSR: Inter-simple sequence repeats. AFLPs: Amplified fragment length polymorphisms. SNPs: Single nucleotide polymorphisms. GBS: Genotyping by sequencing. Tn: repetitive extragenic palindromic elements.

2.1.1.1. Microsatellites: Single tandem repeats (STRs)

Present in eukaryotic genomes, STRs are ubiquitous repeat regions from 1 to 6 base pairs (bp) in length. The units are repeated typically 5-50 times and account for about 3% of the total genome (Buttler, 2012) and are scattered throughout the genome, occurring around every 10,000 nucleotides (Collins et al., 2003). Compared to other molecular markers, STRs are uniquely characterized by their simplicity, abundance, ubiquity, variation, co-dominance and multi-alleles among genomes (Powell et al., 1996). Therefore, during the last 30 years, they have become the forensic markers of choice because of their high variability and easy amplification by PCR (Buttler, 2012).

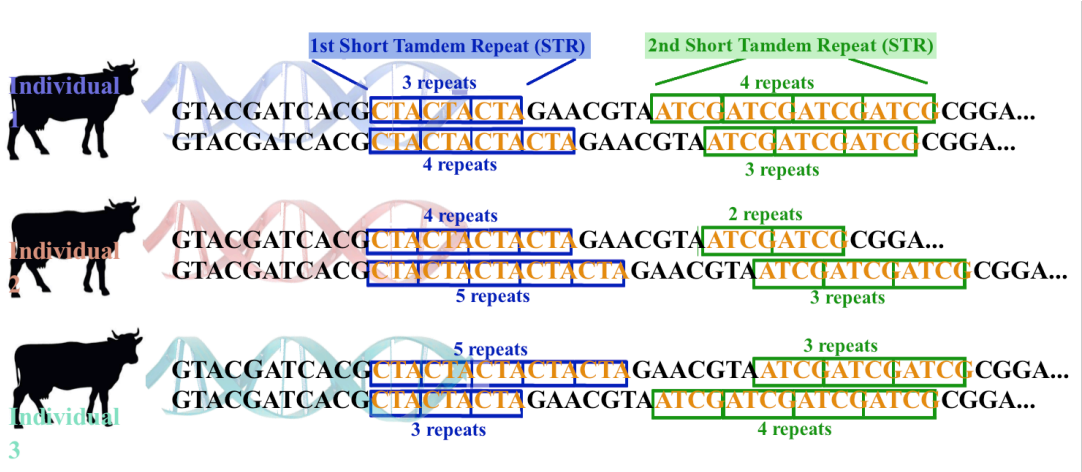


Figure I.11. Example of DNA typing in different individuals.

The number of repeats in STR markers is highly variable among individuals, making these markers highly effective for cattle identification purposes. These markers may present a different number of alleles or repetitions at a specific locus (**Figure I.11.**). Eventually, all the combined genotypes of a set of markers comprise the genetic profile of an individual.

Microsatellites offered a high potential as highly informative markers for studies of genomic variation in important domesticated animal species and hundreds of microsatellites have been characterized in livestock species (i.e., bovine, ovine and porcine genomes). Therefore, microsatellites have been confirmed to be an excellent tool for genetic mapping in various organisms (Vaiman et al., 1994; Ashwell et al., 1996), forensic studies (Van de Goor et al., 2011), and genetic studies for population management and conservation (Cañon et al., 2001; Consenza et al., 2015). They have been used for paternity control in domestic species (Pei et al., 2018), to detect inbred populations (Ginja et al., 2013), in phylogenetic studies (Svishcheva et al., 2020) and for the assignment of individuals to populations (Maudet et al., 2002; Lorenzini et al., 2020) among others.

2.1.1.1.1. Microsatellite characteristics

Microsatellites flancking regions are highly conserved among closely related species (Stalling et al., 1991) and even primers used to amplify a given sequence in one species are used to amplify an analogous sequence in a closely related species (Moore et al., 1991). Amplification of dinucleotide microsatellites in chimpanzees using human primers indicates that microsatellites are highly conserved in terms of chromosomal

location and this conservation may suggest functional conservation. Pépin et al. (1995) typed a panel of 70 bovine microsatellites in goat and other closely related species and found that a total of 41 may be susceptible to amplificate in several species. However, when evolutionarily distant species are compared, conservation is low. Among primates, artiodactyls, rodents and lagomorphs, less than 30% of conserved microsatellite sequences are found (Stallings et al., 1991).

The majority of microsatellites are located in noncoding regions of the genome, such as intergene spacers or introns. Nevertheless, some microsatellite sites located within coding regions of genes are known to cause severe hereditary diseases such as Huntington disease and Fragile X syndrome (Pearson et al., 2005). Although the function of these polymorphisms has not been fully elucidated, many hypotheses have been proposed about its function such as maintenance of chromosome structure (Nordheim & Rich, 1983), DNA packaging during chromosome condensation in meiosis (Gross & Garrad, 1988) or their association with gene regulation and transcription (Hamada et al., 1984). However, they may not have a common function, although they seem to have a common mechanism of evolution (Tautz, 1989).

2.1.1.1.2. Types of microsatellites

STR repeat sequences are named by the length of the repeat unit, being mono-, di-, tri-, tetra-, penta-, and hexanucleotides (Buttler, 2012). The frequency of the different microsatellite sequences differs according to the genome studied, although the most common are dinucleotide repeats, followed by mononucleotide repeats, trinucleotide repeats and the rest to a lesser extent (Beckmann & Saller, 1990). The (CA)n repeats are the most studied so far as they are the most abundant in the mammalian genome. Microsatellites, according to their structure, can be of several types: simple, (containing only one nucleotide motif repeated n times), simple with incomplete units, (containing microvariants), compound, (consisting of two or more stretches of different repetitive motifs) and complex (containing several stretches with variable repetition motif) (Weber, 1990). In cattle, perfect repeats constituted 68% of the sequences, whereas 14.2 repeats have shown to be the average size of the microsatellites (Vaiman et al., 1994).

2.1.1.1.3. Mutation Rate

Mutation rate is defined as the number of mutations that can occur in a single generation. Microsatellite markers mutate at an extremely high rate and are thought to evolve under two different approaches. On the one hand, the stepwise mutation model (SMM) characterized by the addition or deletion of one unit of repeat (Ofta & Kimura, 1973), and the two-phase model (TPM), which allows mutations of 1 repeat unit (one-phase) with probability p and mutations of ≥ 1 unit(s) (two-phase) with probability $1 - p$ (Di Rienzo et al., 1994). Under the SMM and the TPM, a STR is expected to mutate at a constant rate, independently of its repeat length. On the other hand, the infinite alleles model (IAM) accepts a random process, where it is assumed that all possible alleles are equally likely to result from a mutation and originally was proposed for dinucleotides microsatellites (Shriver et al., 1995). However, mutation rates seem to have a great variation among loci. Dinucleotide repeats display the highest mutation rate, while those of tetranucleotide STRs are 50% lower (Chakraborty et al., 1997), and it has been observed that longer microsatellites tend to shorten when a mutation occurs (Calabrese et al., 2001). In humans, a rate between 10^{-2} and 10^{-6} per locus and per generation has been estimated (Li et al., 2002), whereas 10^{-3} to 10^{-4} per locus and per generation in mice (Dallas, 1992). In any case, very high as compared with the rates of point mutation at coding gene loci (approximately 10^{-9} per locus and per generation). Therefore, the high mutation rate of the STR markers makes possible the estimates of within- and between-breed genetic diversity and genetic admixture among cattle breeds. However, the mutation rates of STR loci are approximated, and they should be carefully considered since an incorrect understanding of the data which further lead to erroneous interpretation in parentage and kinship testing (Segurel et al., 2014).

2.1.1.1.4. Nomenclature of microsatellites

In order to make possible interlaboratory reproducibility and comparisons, the animal genetics community agreed a common nomenclature based on the measured length of PCR amplicons. In cattle, this allele nomenclature is internationally regulated by the International Society for Animal Genetics (ISAG) - FAO Advisory Group on Animal Genetic Diversity (FAO, 1993). Nevertheless, it has major limitations due to the lack of effectiveness to compare data and the little overlap in marker choice or different electrophoretic platforms used among laboratories. Allele nomenclature based on number of repeats has been promoted for human genotyping, however few efforts have

been made to adapt the same allele nomenclature to a number of widely used important animal species such as cattle, horses and pigs, although some STR nomenclature proposal has been proposed for dogs and cats (Eichmann et al., 2004; Menotti-Raymond et al 2005; Berger et al., 2018). In cattle, the first proposal for a repeat-based nomenclature was made by Van de Goor et al. (2009) for 16 microsatellite markers and considering a repeat-based alleles nomenclature observed from a dataset covering 22 cattle breeds. Nevertheless, the length of PCR amplicons is the standard nomenclature commonly used during the ISAG organized interlaboratory comparison tests, and therefore the preference by many laboratories.

2.1.1.1.5. Cattle microsatellites

2.1.1.1.5.1. Microsatellite marker panels

In 1993, a group of experts from the ISAG established a series of recommendations, and assist the selection of most suitable microsatellites based in their properties for the analysis of genetic distance and the characterization of different species of zootechnical interest (FAO, 1993). According to these criteria, FAO recommended a list of microsatellites described in a Secondary guidelines for the measurement of domestic animal diversity (MoDAD) (FAO, 1998), although the results of a survey conducted by FAO on genetic diversity studies in domestic animals showed that this list was not widely used in bovine studies (Baumung et al., 2004). Therefore, a new microsatellite marker panel was recommended for livestock animals, including cattle (FAO, 2004). In 2007, *The State of the World's Animal Genetic Resources for Food and Agriculture* provided the first report of the global assessment of the status and trends of AnGR in 169 countries, including the institutional and technological capacity to manage these resources (FAO, 2007a). It was concluded that the diversity of AnGR was decreasing and, as a global response, a Global Plan of Action was proposed to enhance the sustainable use and conservation of AnGR (FAO, 2007b). The characterization of AnGR became one of the first Strategic Priority in order to properly assess the value of breeds and to guide decision making in livestock development and breeding programmes. Therefore, new guidelines for the *Molecular characterization of animal genetic resources* have been developed to help countries to plan and implement effective analyses of the genetic diversity in the need to acquire international technical standards and protocols for characterization of AnGR (FAO, 2011).

Table I.2. Commercial and recommended (FAO and ISAG) STR panels used.

FAO 2004	ISAG conference 2006	ISAG conference 2008	Stockmarks ®	Bovine Genotypes Panel 1.2	Bovine Genotypes Panel 2.2	FAO 2011
BM1824	BM1824	BM1824	BM1824	BM1824		BM1824
BM2113	BM2113	BM2113	BM2113	BM2113		BM2113
ETH03		ETH03	ETH03	ETH03		ETH03
ETH010	ETH10	ETH10	ETH10	ETH10		ETH010
ETH225	ETH225	ETH225	ETH225	ETH225		ETH225
INRA023	INRA023	INRA023	INRA023	INRA023		INRA023
	SPS115	SPS115	SPS115	SPS115		SPS115
	TGLA122	TGLA122	TGLA122	TGLA122		TGLA122
	TGLA126	TGLA126	TGLA126	TGLA126		TGLA126
	TGLA227	TGLA227	TGLA227	TGLA227		TGLA227
BM1818		BM1818		BM1818		BM1818
		TGLA053	TGLA053	TGLA053		TGLA53
CSSM66					CSSM66	CSSM66
CSRM60					CSRM60	CSRM60
ILSTS006					ILSTS06	ILSTS006
HAUT27						HAUT27
HEL01						HEL01
						INRA005
						INRA037
INRA063						INRA063
ETH152						ETH152
HEL09						HEL09
						ETH185
HEL05						HEL05
HEL13						HEL13
						INRA032
MM12						MM12
						HAUT024
ILST005						ILST005
INRA035						INRA035
INRA052						
MM8						
ETH185						
ILSTS030						
ILSTS034						
ILSTS033						
ILSTS011						
ILSTS054						
					SPS113	
					RM067	
					MGTG4B	

This report proposed a panel of 30 microsatellite markers for cattle (**Table I.2.**). During all this process of decission making and proposing markers, different markers have been used in the scientific literature, which have somehow complicated the comparison of STR data.

In cattle forensic genetics, the ISAG suggested to use a panel of 9 loci for cattle parentage analysis (ISAG Conference, 2006), in which three new loci were included later (ISAG Conference, 2008). Since that, several commercial panels, that include ISAG markers, have been developed such as the 11-STR panel of StockMarks® (Applied Biosystems, Foster City, CA, USA) or the 12-STR core panel (Bovine Genotype™ Panel 1.2, Thermo Scientific, Waltham, MA, USA). Finally, six additional markers were included in a complementary panel (Bovine Genotype™ Panel 2.2, Thermo Scientific, USA), to be used when more STR loci are required.

2.1.1.1.5.2 Microsatellite studies in cattle

According to one of the first molecular studies considering 37 European cattle breeds using blood group and serum protein polymorphisms, overall relationships among breeds reflected their geographical origin and common ancestry rather than the agricultural use for which the breeds have been selected (Blott et al., 1998). However during the last 200 years, the requirements of herd books have led to the genetic isolation of many cattle breeds (Maudet et al., 2002). Recently, the data using STR markers have generated information about the history of these individual breeds, but also related to the genetic constitution of a breed and its degree of isolation from other breeds.

In the 20th century, the usefulness of STR typing allowed the analysis of genetic structure and population genetic variation within and between Italian (Ciampolini et al., 1995), French (Moazami-Goudarzi et al., 1997), and Swiss cattle breeds (Schmid et al., 1999) or, in a more general perspective, within and among European cattle breeds (Machugh et al., 1994). A random panel of 20-STR markers has also shown to be helpful for investigations of gene flow and admixture in African cattle populations where the introgression of zebu (*Bos indicus*) specific alleles in African cattle (*Bos taurus)* provided a high-resolution perspective on the hybrid nature of African cattle populations (Machugh et al., 1997).

The geographical location of Iberian Peninsula is considered a contact region between

the African and European continents through the Strait of Gibraltar. And throughout history, humans inhabiting the Iberian Peninsula have contributed to the wide variety of autochthonous cattle breeds existing in this territory. However, the origin of present day Iberian cattle breeds has been controversial. African-zebu (*Bos indicus*) mtDNA haplotypes observed in Iberian sourthern breeds suggested an African influence reflecting an intercontinental admixture in the initial origins of Iberian breeds (Cymbron et al., 1999). However, Beja-Pereira et al. (2003) could not detect any strong evidence for an African-zebu genetic influence in the Iberian cattle breeds using 20-STR markers. A genetic study among 18 local cattle breeds from Spain, Portugal and France using 16-STR markers showed a variation among breeds sufficiently high and assigned individuals to their breed of origin with a probability of 99% (Cañon et al., 2001), but the introgression of African-zebu alleles was not studied. In a later study by Cymbron et al., (2005), a 19-STR marker analysis evidenced two distinct cattle migration routes from the Near East, and also demonstrated that Mediterranean cattle breeds may have more recent input from both the Near East and Africa.

Molecular characterization of AnGR has recognized to be a logical approach to conservation and therefore, during the last years, several genetic studies have used STR markers to chracterize Spanish autochthonous cattle breeds (Rendo et al., 2004; Martin-Burriel et al., 2007; Martin-Burriel et al., 2011; Sanz et al., 2014). Genetic data of the autochthonous Pirenaica breed has shown a heterozygosity value of 0.628 using 16-STR panel (Cañon et al., 2001) or 0.688 using 11-STR (Rendo et al., 2004), which are higher values than the ones reported by Martin-Burriel et al., (1999) (0.617) probably due to sampling or panel selection even though 30-STR panel were genotyped. More recently, a 10-STR panel was used in Pirenaica breed for traceability purposes (Arana et al., 2011), and even if the ISAG a 12-STR panel has been also widely used in parentage and identity testing, it showed poor discrimination power among several European breeds. Therefore, an increase in the amount of genotyped STRs has been suggested for forensic purposes in cattle (Van de Goor et al., 2011). The Terreña breed has been poorly studied, but it showed a heterozygosity value of 0.747 with an 11-STR analysis (Rendo et al., 2004). Nonetheless, new genetic studies about Terreña might be neccesary to clarify the current diversity of this endangered local breed.

Over the last 50 years, the prevalent livestock model in Spain has experienced a radical transformation and more productive breeds have been introduced looking for productive improvement. Charolais, Limousin, Blonde d'Aquitaine and Salers are some of the most relevant breeds in the close France and have also been imported to Spain and

the Basque region. Moazami-Goudarzi et al. (1997) suggested that even 30 microsatellites are used, simulation studies might be required to adequately distinguish closely related French breeds. However, according to Maudet et al. (2002), in Charolais, Limousin and Holstein breeds, among others, assignment tests could reliably identify individuals to its breed of origin with a high certainty (99.9 %) using a 23-STR panel. More recently, a genetic study using only a 16-STR panel showed a clear differentiation among Limousin, Blonde d'Aquitaine and Salers, and exhibited a 9 % of the total variation owing to breed differences (Amigues et al., 2010). Previously, Cañon et al. (2011) and Beja-Pereira et al. (2003) showed that genetic similarity among neighboring Iberian breeds is mainly caused by gene flow, and even French breeds (Aubrac, Gasconne and Salers) were included, they were not deeply studied. However an extensive genetic characterization of Salers breed raised in Spain might be necessary to further study its genetic diversity since its introduction in Spain and the Basque region. On the other hand, highly selected and intensively managed Holstein-Friesian has now grown in number at the expense of local cattle breeds (European Cattle Genetic Diversity Consortium, 2006), becoming a extensively studied breed on a regional or national scale (Cymbron et al., 2005; Ginja et al., 2009; Amigues et al., 2010; Heo et al., 2014; Wang et al., 2014; Brenig et al., 2016; Agung et al., 2019). In this regard, it is important to note that the Holstein-Friesian raised in the Basque region had not been characterizated and, therefore, its worldwide extension and presence in this region sustain the need to study its population and genetic relationships with other breeds.

2.2. IMPROVEMENT THROUGH GENETICS

In the past, the selection of individuals was based solely on morphological phenotypes and following the producer's intuition. Nowadays, genetic selection is an on-growing strategy to improve production as a response to new markets in livestock industry, becoming the most efficient and practical option to improve meat quality traits. Every breed has a wide range of variability in its genetics. Therefore, efforts to improve the genetics associated with meat quality must be based on individual animals performance, not the reputation of a breed or general trends for a breed. In this sense, genetic studies related to meat quality become a necessary step into the marker assisted selection, and to provide valuable knowledge to producers interested in raising local pure breeds and selection of individuals with superior traits.

2.2.1. IMPROVEMENT POLYMORPHISMS

The most used methods in population genetics laboratories are the PCR systems based on STRs both for individual identification and kinship testing, due to their higher power of discrimination, multiplex capability and high speed of analysis (Buttler, 2012). Apart from STRs, in the last 20 years, other kind of markers have been studied for genetic analysis such as the mtDNA, SNP and insertion-deletion polymorphism (INDEL). However, the beginning of the 21st century has provided new molecular biology technologies which enabled the discovery of new and more discriminative markers, as well as the development of new molecular techniques able to genotype higher number of makers in a single PCR reaction. In the last two decades, a massive progress has been made in molecular biology since the development of next-generation sequencing (NGS) technologies. In essence, the principal innovations of NGS platforms have focused on massive parallel chemistry, ultrahigh-resolution optics and powerful computational methods. These revolutionary technological advances have drastically reduced the cost and shortened the time for results. Nowadays, science is facing another challenge related to the huge amount of information obtained and the problem of millions of misleading results, with the challenge of implementing data storage and processing methods (Wong, 2013; Van Dijk et al., 2014).

The investigation of some polymorphisms, specially single nucleotide polymorphisms (SNPs), has enabled the identification of quantitative trait loci (QTL) and the implementation of genomic prediction in many livestock species. Many genome-wide association studies (GWAS) have been performed using this SNP information to identify QTLs (Cole et al., 2011; Buzanskas et al. 2017; Vineeth et al. 2020). However, significantly associated regions include large genomic areas containing numerous candidate genes, which makes it challenging to identify the causative mutation itself. Therefore, other strategies have performed genetic analyses in specific candidate genes related to metabolic pathways that have already demostrated to affect traits of economic interest. These strategies based on the resequencing of candidate genes and genotyping by sequencing (GBS) have provided evidence of potential causative mutations related to quality traits. Causative mutations could be used as molecular markers in early marker-assisted selection (MAS) in beef cattle breeding programs (Rincon et al. 2012; Zheng et al. 2019; Bordbar et al. 2020).

Livestock and meat scientists have intensively committed large efforts searching for polymorphisms and causal mutations with remarkable effect on quality traits. High-

throughput multiplex polymorphism analysis, as an approach to QTL mapping, has shown a great number of QTLs and genetic associations in cattle. According to the Cattle Quantitative Trait Locus Database, there are 163,725 cattle QTL / associations, related to 185 cattle genes and released from 1,069 publications. This data showed 685 different cattle traits, while 1,201 of the QTLs are associated with the beef fatty acid content (CattleQTLdb, 2021).

2.2.1.1. Insertion-Deletion polymorphisms (INDEL)

In recent years, more attention has been paid to INDEL, as the second main form of genomic variation, and great contributions have been made to investigate on genetic and phenotypic diversities in human and cattle (Grobet et al., 1997; Kunieda et al., 2005; Montgomery et al., 2013). Compared to SNP and Copy Number Variants (CNV) molecular markers, INDEL is a relatively simple marker, time-saving and widespread in the genome, which could be effectively applied in animal biotechnology and marker assisted selection breeding (Peng et al., 2019). Certain studies, in humans and chimpanzees, evidenced that INDELs, instead of SNPs, were the major source of evolutionary change (Britten, 2002; Anzai et al., 2003), and potentially multi-allelic and co-dominant, offering more genomic information than the usually bi-allelic SNPs (Lv et al. 2016). Finally, genotyping of INDEL markers is technologically less expensive, whereas SNP detection usually requires costly chemicals and equipment.

Several studies have confirmed the presence of INDEL markers in cattle related to horn growth and polledness (Wiedermar et al., 2014), fertility (Sasaki et al., 2015), body measurement traits (Jin et al., 2018), and growth traits (Xu et al., 2018, Wu et al,, 2019). INDELs can also produce changes in the reading frame of a gene or modify the number of amino acids in a protein, but also affect gene expression levels. INDEL markers were found to affect backfat thickness (Ren et al., 2012) and fat deposition (Zang et al. 2016; Crespo-Piazuelo et al., 2019) in pigs through the alteration of gene expression, underlining the importance of these variants for livestock production. In cattle, INDELs have been associated with higher content of monounsaturated fatty acids (MUFAs) and triglycerides (Xu et al., 2013), whereas QTL mapping has also confirmed the association between an INDEL and the fatty acid composition (Kawaguchi et al., 2021).

3. MEAT QUALITY

Meat quality is an important trait and its various characteristics (i.e., fat content and composition, tenderness, colour, juiciness/water-holding, flavour) are of great importance considering their effect in the economic value of meat and derived products. Bovine adiposity is not only a component of carcass composition, but also a determinant of carcass quality and economic value, considering its positive contribution to the appearance, texture, firmness, caloric value, and shelf-life of red meat products (Dodson et al., 2010). Lipids also play an important role in meat quality and much of the species-specific flavour of meat is derived from adipose tissue.

Meat composition has a great impact on human nutrition, as it is a source of high quality protein, vitamins B6 and B12, niacin, iron, and zinc (Bender, 1992). However, beef fat has gained the reputation of being less healthy due to the presence of saturated fatty acids (SFA) such as myristic (14:0) and palmitic (16:0) acids, which has been linked to cardiovascular pathologies (Keys et al., 1974). Whereas experimental studies have showed that several components (i.e., heterocyclic amines, polycyclic aromatic hydrocarbons and *N*-nitroso compounds) of red and/or processed meat may impact on cancer risk and promote carcinogenesis (Abid et al., 2014). Therefore, the causes of the increase in cardiovascular disorders and obesity in the population are of growing concern (WHO, 2004, IARC, 2015; Bouvard et al., 2015).

Adipose tissue like backfat or subcutaneous fat are mostly formed by neutral lipids mainly composed by fatty acids (FA) coming from peripheral circulation directly from the digestive system. Therefore, FA composition of the neutral lipid fraction plays an important role in the composition of meat, whereas its regulation by lipogenic genes offer multiple approaches for a better understanding of adipogenesis and lipid metabolism.

Overall, dietary recommendations advise replacing SFA with polyunsaturated fatty acids (PUFA) and increasing the intake of n-3 PUFA (FAO/WHO, 2009), while other molecules such as conjugated linoleic acids (CLA), showed that grilled beef had an inhibitory effect on inhibited mutagenesis and carcinogenesis (Pariza et al. 1979, Pariza et al. 2000). The discovery that CLA inhibited carcinogenesis in several animal models has led to numerous investigations into the biochemical mechanisms of action of CLA and its beneficial physiological health effects (Ip et al., 1995; Gavino et al., 2000; West et al., 2000; Pariza et al., 2002; Tsuzuki et al., 2004) although the effect of individual CLA

isomers still needs to be clarified and, therefore, more methodologically appropriate (correct separation of CLA isomers) studies are needed. Even if these CLA can be found in small proportions in vegetable oils, their concentration is particularly high in ruminant animals being the main dietary CLA source, including the 9*c*,11*t*-18:2 isomer (Kramer et al., 1998), which is known for its potential health benefits (Shingfield et al. 2014). From this, it is possible to deduce that the best dietary source of CLA is the consumption of meat and dairy products from ruminants. In this regard, many studies have investigated the beneficial effects of CLA in reducing the incidence of atherosclerosis (McCarthy et al., 2013), cancer (Islam et al., 2008), stimulation of immune function (Bhattacharya et al., 2006) and obesity (Wang & Lee, 2013), whereas the benefits of vaccenic acid (VA; 11*t*-18:1) and ruminant trans fats (Alves et al., 2021) and branched-chain FAs (BCFA) (Cai et al., 2013) have also been reported. Therefore, there is a great interest in increasing the content of several FAs in meat and dairy products because of health beneficial properties.

3.1. FATTY ACID COMPOSITION OF BEEF

Ruminants have a unique digestive system formed by a four-compartment stomach (rumen, reticulum, omasum and abomasum), which allows them to ferment feedstuffs and dispose a better use energy from fibrous plant material than monogastric animals. In cattle, as well as other ruminants, diet can modify significantly the rumen microbiota and, therefore, the FA composition of ruminant-derived food products (Shingfield, & Griinari, 2007, Aldai et al., 2013, Bessa et al., 2015, Doreau et al., 2015). However, other minor FA can be also *de novo* synthesized by Stearoyl-CoA Desaturase (SCD, EC 1.14.19.1 or Δ9-desaturase) activity or by elongation and desaturation of circulating FAs to form long-chain polyunsaturated FA (PUFA) (Leonard et al., 2004).

In ruminants, isomeric forms of 18:1, 18:2, 18:3 FA and CLAs are formed during biohydrogenation and are considered intermediate compounds of the biohydrogenation processes (Kramer et al., 2004; Destaillats et al., 2005; Chilliard et al., 2007). CLAs are also produced in the rumen by biohydrogenation of microbiota, where the linoleic acid (9*c*,12*c*-18:2) is transformed to rumenic acid (9*c*,11*t*-18:2) (**Figure I.12.**). VA (11*t*-18:1) can also be converted to its corresponding CLA isomer (9*c*,11*t*-18:2) (Bauman et al., 1999), and can also be endogenously originated in the animal tissue by the enzyme SCD (Griinari et al., 2000).

VA (11*t*-18:1) has been recognized as the main intermediate compound formed during biohydrogenation in ruminants under diets with low concentrate to forage ratio (C/F), typically from extensive or semi-extensive production systems (Bauman, & Griinari, 2003). In contrast, in intensive production systems based on concentrates with high C/F ratio, 10*t*-18:1 production is increased instead of vaccenic acid (Alves et al., 2014; Aldai et al., 2013; Alves et al., 2021) In addition, the 10*t*,12*c*-18:2 produced from biohydrogenation, is reported to inhibit lipogenesis, through the decrease of mRNA abundance for enzymes involved in the uptake, synthesis, transport, desaturation and esterification of FAs in mammary gland, being the main responsible for milk fat depression syndrome and other metabolic problems in lactating ruminants (Bauman, & Griinari, 2003; Russell, & Rychlik, 2001).

After biohydrogenation, the lipids available for absorption in the duodenum include mainly SFAs, such as stearic acid (18:0) and palmitic acid (16:0), but also biohydrogenation intermediates (11*t*-18:1 and other *trans*-MUFAs). Subsequently, FAs are transported into the cells to be stored in the form of triacylglycerols in lipid dropets. In adipocytes, the *SREBP* key genes are activated by proteolytic cleavage regulated by insulin, glucagon, esterols and liver X-activated receptors (LXRs) (Eberle et al., 2002; Horton et al., 2002; Horton et al., 2003). The SREBP/SCAP/INSIG protein complex is retained in the endoplasmatic reticulum and, upon appropriate conditions, SREBP/SCAP complex cleavages and moves to the Golgi apparatus. Later, the site 1 and site 2 proteases cleaves mature SREBP, so it can be translocated to the nucleous (**Figure I.12.**). The FA biosynthesis occurs by several pathways activated by *SREBP1*, including different enzymes such as ATP-citrate lyase (ACL), acyl-CoA synthethase (ACS), acetyl-CoA carboxylase (ACAC), the fatty acid synthase (FASN), fatty acid elongases (ELOVL), stearoyl-CoA desaturase (SCD) and fatty acid desaturases (FADS) (Urrutia et al., 2020) (**Figure I.12.**). Acetate produced from the tricarboxylic acid cycle is the principal precursor of *de novo* lipogenesis, whereas the hidrogen sources come from glucolysis. *De novo* FAs and those obtained from the diet (after biohydrogenation) are transformed by desaturation (SCD and FADS) and elongation (ELOVL) enzymes. Among them, the key SCD is a limiting enzyme as main step to produce major MUFA of membrane lipoproteins, tryacylglicerols and cholesterol esters. Although, more than 70 % of essential PUFAs (18:2n-6 and 18:3n-3) are transformed by biohydrogenation in the rumen (Jenkins et al., 2008). They can also be transformed by desaturation and elongation to long-chain PUFAs, although this process is inefficient and most long-chain PUFAs are derived from the diet.

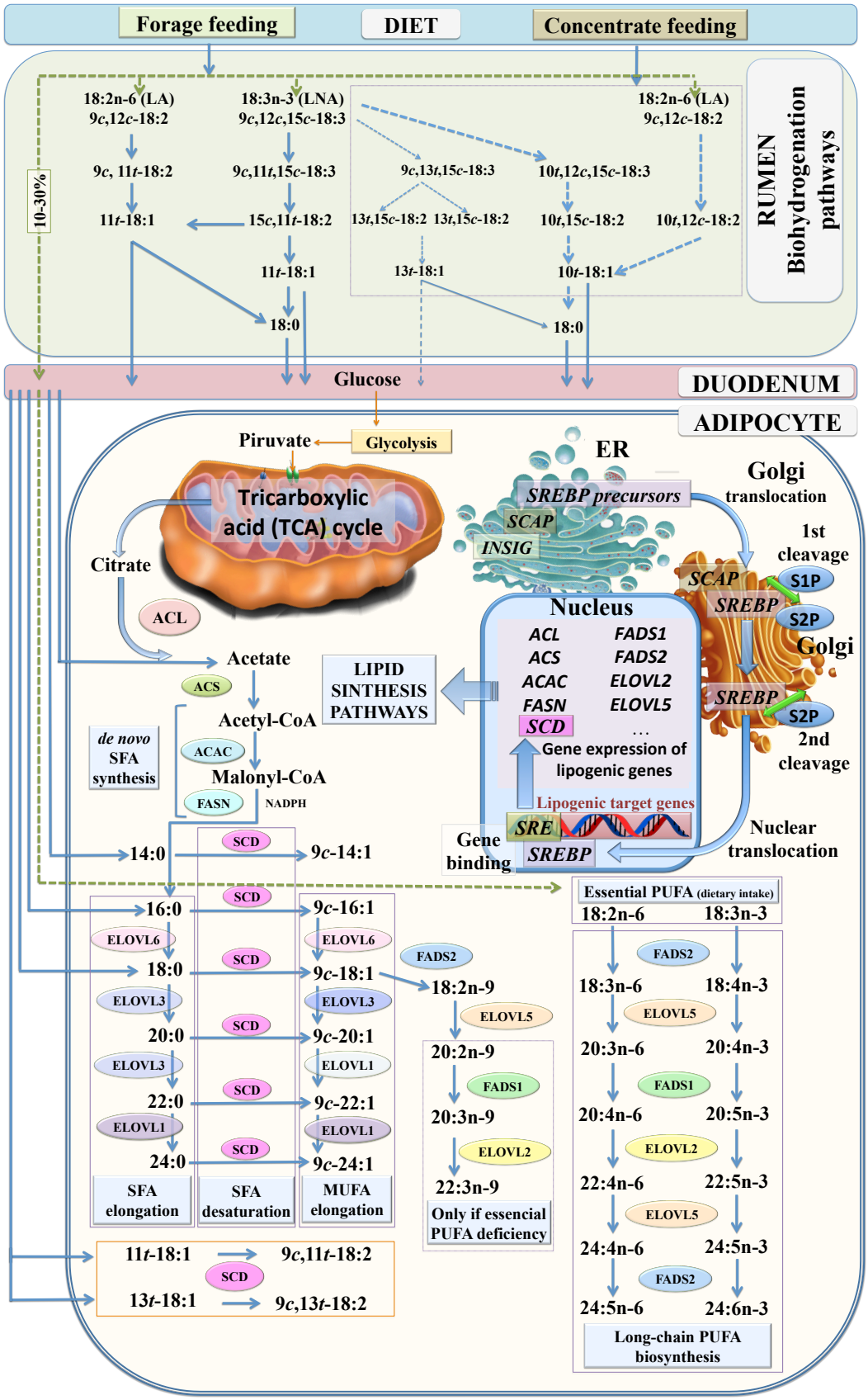


Figure I.12. Schematic representation of the metabolic pathways involved in the biohydrogenation of PUFA in the rumen and the synthesis of fatty acids in adipose tissue. In rumen, continuous arrows describe major pathways, discontinuous green lines describe minor

pathways, and discontinuous blue lines described the altered pathway proposed by Alves et al. (2021). Adapted from Eberle et al., (2002); Horton, (2003); Bauman & Griinari, (2003); Jenkins et al., (2008); Shinfield & Wallace, (2014); Sassa & Kihara, (2014); Urrutia et al., (2020). ACAC: acetyl-CoA carboxylase; ACL: ATP-citrate lyase; ACS: acyl-CoA synthetase; LNA: α-linolenic acid; ER: Endoplasmatic Reticulum; ELOVL1, 2, 3, 5 and 6: fatty acid elongase 1, 2, 3, 5 and 6; FA: fatty acid; FASN: fatty acid synthase; FADS1 and 2: fatty acid desaturase 1 and 2; INSIG: insulin-induced protein; LA: linoleic acid; SCAP: SREBP cleavage activating protein; S1P: site 1 protease; S2P: site 2 Protease; SCD: stearoyl-CoA desaturase; SREBP: sterol regulatory element-binding protein; SRE: sterol regulatory element.

3.1.2. RELATIONSHIP OF FATTY ACID COMPOSITION AND LIPOGENIC GENES

Many lipogenic genes have been identified as prospective targets for the regulation of FA synthesis, and therefore, there is an increased interest to elucidate the associations between lipogenic genes and FA composition. In animals' cells, FA synthesis takes place in the cytosol, in contrast with β-oxidation, the degradation pathway that happens in mitochondria. The FAs are *de novo* synthetized from acetyl-CoA, malonyl-CoA and NADPH through an enzyme complex called FA synthase (FASN), with the main product as palmitic acid (16:0) (Maier et al., 2006). *De novo* synthesized 18:0 or even longer FA chains are produced from 16:0 by FA elongases, whereas if a *cis* double bound is introduced to SFAs by SCD enzyme MUFAs are produced (**Figure I.12.**).

During adipocyte differentiation, the increase in FA synthesis arises from increased rates of some of the lipogenic enzymes activity such as FASN (Student et al., 1980), suggesting also an increased mRNA abundance. Therefore, it is evident that to understand the genetic mechanisms responsible for changes in FA composition, it could be necessary to identify genes whose expression is activated during FA synthesis.

Among transcription factors and nuclear receptors, peroxisome proliferator-activated receptor gamma (PPARγ) and transcription factors designated as sterol regulatory element binding proteins (SREBP) are considered master regulators of lipid metabolism (Zhao et al., 2010). Lipogenesis is controlled at the transcriptional level by the family of SREBPs (Brown & Goldstein, 1997) and its association with healthy FA profiles in milk and meat are of permanent interest (Bauman et al., 2011; Ladeira et al., 2016). SREBP transcription factors are regulated at three major levels: (1) transcription, (2) proteolytic cleavage of SREBP precursors, and (3) post-translational modification of nSREBPs (Eberle et al. 2002). Directly, SREBPs activate the expression of more than 30 genes dedicated to the synthesis and uptake of cholesterol, FAs, triglycerides, phospholipids (Horton et al. 2002), and regulate gene transcription activation by binding to the Sterol

Regulatory Element (SRE) sequences present in the promoter of downstream genes such as *SCD* genes (Shimano, 2001; **Figure I.12.**). Therefore, lipogenic gene expression and polymorphism-association analyses might be key to elucidate the transcriptional cascades modulating the adipogenesis.

3.1.2.1. *SREBP* genes ad fatty acid composition

Among the different genes involved in lipid metabolism, *SREBP* family have shown different roles in the adipocyte differentiation (Briggs et al., 1993) and the lipogenesis (Brown & Goldstein, 1997). In mammals, there are two SREBP isoforms (SREBP-1a, SREBP-1c) encoded by *SREBP1* gene (located in bovine chromosome 17) by alternative splicing of the first exon, and a SREBP-2 isoform transcribed by a different *SREBP2* gene (located in chromosome 5) (Horton et al., 2002). SREBP-1c isoform is the most abundant in tissues with active *de novo* FA synthesis, such as liver and adipose tissue, and it is predominantly regulated at transcription, post-translation, and protein stability level (Osborne et al., 2000). In fact, it has also been proposed that the protein SREBF-1c could be regulated by the degree of saturation of lipids (Worgall et al., 1998), whereas the *SREBP1* gene showed to inhibit lipogenesis mediated by PUFA (Jump et al., 2005). Graugnard et al., (2010) also reported that *SREBP1* gene could be nutritionally regulated, showing a greater *SREBP1* expression in Angus steers fed with high-starch diet compared to a low-starch steers group. Moreover, the implication of *SREBP1* in the overall regulation of milk fat synthesis have also been demostrated. *In vivo* studies have shown the involvement of *SREBP1* during diet-induced changes in mammary lipogenic gene expression from lactating dairy cows (Angulo et al., 2012), while *SREBP1* was inhibited in lactating cows fed with a low-forage/high-oil diet or infused with 10*t*,12*c*-18:2 (Peterson et al., 2004; Harvatine & Bauman, 2006; Gervais et al., 2009).

Taking into account the importance of *SREBP* genes in the lipogenic pathways, the characterization of allelic variation in this genes might be helpful as a tool for marker-assisted selection. In this regard, an INDEL polymorphism (rs133958066 or 84bp-indel) in the intron 5 of *SREBP1*, was firstly identified and associated with MUFA content and lower melting point of intramuscular fat of Japanese black cattle (Hoashi et al., 2007). The gene frequencies of INDEL polymorphism of the *SREBP1* gene were found to be different depending on breed. INDEL polymorphism was abscent in Hereford, Holstein-Friesian or in three zebu cattle breeds (*Bos indicus*) of south Asian countries (Myanmar, Laos and Cambodia) suggesting that *Bos indicus* animals have not been improved for

beef and milk related traits (Kaneda et al., 2011). In addition, this 84bp-indel polymorphism showed very low frequencies in the S allele of Anatolian Red cattle (Öztabak et al., 2013). The 84bp-indel showed no effect in the FA composition, although another SNP in *SREBP1* (rs41912290) revealed significant effect on marbling score, MUFA and 9*c*-18:1 content of muscle fat in commercial Korean Hanwoo cattle (Lee et al., 2013). In Japanese black cattle, 84bp-indel was not associated with FA composition of the *longissimus thoracis* muscle (Matsuhashi et al., 2011). However in this study, 2 polymorphisms, in *FASN* and *SCD* genes, separately influence the FA composition. They particularly observed that in the polymorphism V293A of *SCD*, firstly reported by Taniguchi et al. (2004), when allele V was substituted for allele A, SFA content (14:0, 18:0) was reduced, but MUFA content (9*c*-14:1, 9*c*-18:1) was increased.

On the other hand, S allele of *SREBP1* was observed at a low frequency in Fleckvieh bulls, but LS genotype was associated with higher 9*c*-14:1 profile in subcutaneous fat compared to LL genotype (Barton et al., 2010). The polymorphism 84bp-indel has been significantly associated with the concentration of 9*c*-17:1 in adipose tissue of Angus and Charolais based crossbred commercial steers (Han et al., 2013). In addition, LL genotype of *SREBP1* was signifcantly associated with higher content of palmitoleic acid (9*c*-16:1), triglycerides and the 9*c*-16:1 desaturation index (DI), but lower content of stearic acid (18:0) and SFA contents compared to those with the LS genotype in Simmental bulls (Xu et al., 2013). The 84bp-indel polymorphism has also been associated with an increase in the body weight at 18 to 24 months of age in several chinese cattle breeds (Huang et al., 2011). Additionally, QTL studies, genome-wide association scans (GWAS) and functional genomic analyses indicated that *SREBP* genes are key factors regulating milk fat synthesis (Harvatine et al., 2009; Conte et al., 2010). And in this line, Rincon et al. (2012) studied several lipogenic genes and observed that a SNP (rs41912290) in *SREBP1* accounted for 40 % of the phenotypic variance in Holstein cows with low milk fat syndrome, whereas several polymorphisms in the *SCD5* and *INSIG2* genes were the most representative markers associated with SFA/UFA ratio in milk. Furthermore, the long genotype (LL) of 84bp-indel has been related with a higher fat content in the first lactation and a higher protein content in the first three lactations of Jersey cattle (Proskura et al., 2017).

3.1.2.2. *SCD* genes ad fatty acid composition

In the subcutaneous and intramuscular fat depots of beef cattle, the majority of SFA conversion to MUFA is catalyzed by SCD enzyme (St John et al., 1991). Therefore, the SCD enzyme had been proposed as a new regulator of body energy homeostasis (Paton & Ntambi, 2009), and is one of the most promising candidate genes to explain variability in FA composition. Several studies have shown that the differences in MUFA percentage were correlated with SCD1 activity in cattle (Sturdivant et al., 1992; Yang et al, 1999; Laborde et al., 2001). Although, *SCD1* expression is regulated by *SREBP1*, other regulatory elements including *INSIG* (insulin-induced protein) and *SCAP* (cleavage of activation by *SREBP* protein) among others can also affect its expression (Mauvoisin & Mounier, 2011). In addition, *SCD1* expression is regulated by energy content, but also exhibits a differential response to high-fat and high-carbohydrate diets. A high PUFA diet reduces a 75 % of the *SCD1* mRNA expression (Jones et al., 1996), but also 10*t*,12*c*-18:2 isomer of CLA suppresses *SCD1* expression (Jaudszus et al., 2010; Choi et al., 2000), suggesting that 10*t*,12*c*-18:2 and other PUFA negatively regulate *SCD1* expression in adipocytes. Considering that 10*t*,12*c*-18:2 isomer is generally produced by shifted rumen biohydrogenation and its accumulation can depress adipogenesis, this FA can effectively block the conversion of vaccenic acid (11*t*-18:1) to rumenic acid (9*c*,11*t*-18:2). Any feeding practice that increases the formation and absorcion of 10*t*,12*c*-18:2 will reduce the expression *SCD*, consequently decreasing the formation of 9*c*,11*t*-18:2 and other MUFAs (Smith et al. 2009). In contrast, an SFA-rich diet has shown an increase *SCD1* expression in murine adipose tissue (Nikonova et al., 2008).

When different animal tissues were compared, *SCD* gene expression and SCD catalytic activity is significantly higher in bovine, ovine and porcine adipose tissues than muscle, liver or intestinal mucosa (**Figure III.13.**; St John et al., 1991; Chang et al., 1992; Klingenberg et al., 1995; Archibeque et al., 2005), and it is elevated in adipose tissues containing a high concentration of MUFA (Chung et al., 2007; Brooks et al., 2011). In fact, subcutaneous adipose tissue has approximately twice the SCD activity of intramuscular adipose tissue (Archibeque et al. 2005), that agree with the relatively higher MUFA content in subcutaneous compared to intramuscular adipose tissue (Brooks et al. 2011). However, when a comparison among breeds was performed, even a positive relationship was observed between *SCD1* gene expression and FA composition, breeds with higher MUFA content did not show higher *SCD* gene expression (Chung et al., 2007).

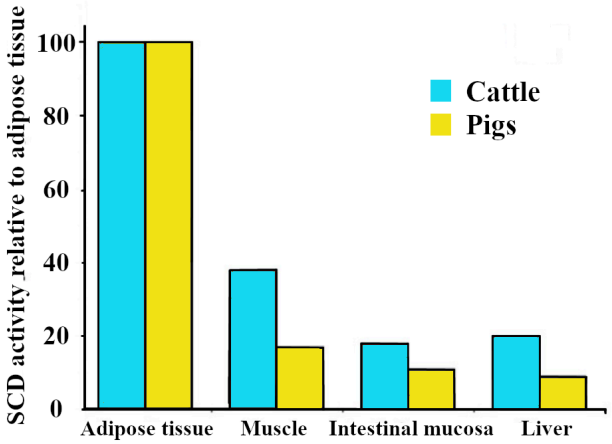


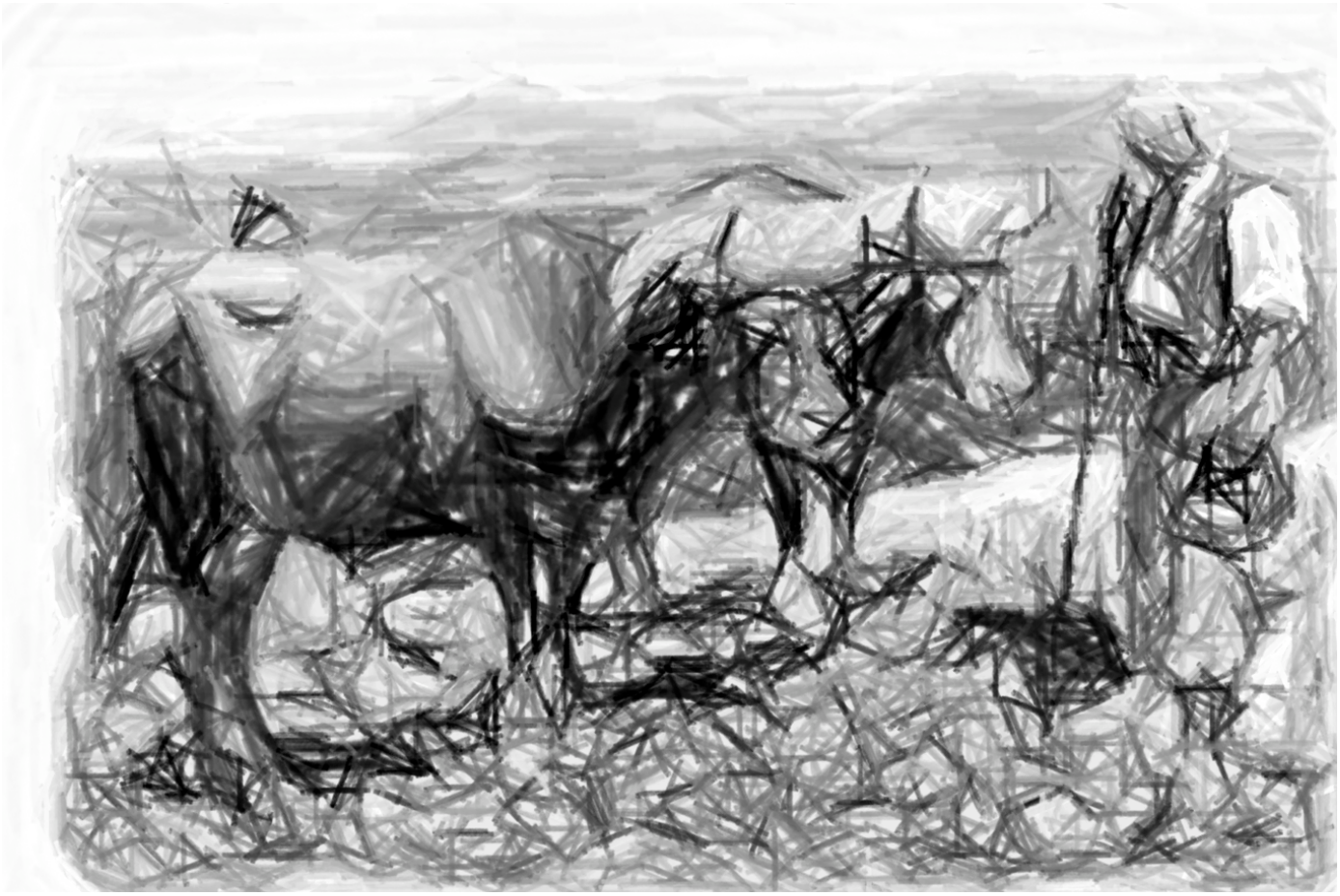
Figure I.13. SCD activity in several tissues (adipose tissue, longissimus muscle, duodenal intestinal tissue and liver) of cattle and pigs (modified from Smith, 2013).

Genetic polymorphisms in the *SCD1* gene have been associated with fat distribution and insulin sensitivity (Gong et al., 2011), whereas an SNP in *SCD* gene has been recently associated with a decreased atherosclerosis in humans (Liu et al., 2020). In cattle, an extensively investigated SNP in the position 10329 of the *SCD1* gene encodes for a different codon (Val/Ala variant), and it has been reported to contribute to a higher MUFA content in the intramuscular fat of Japanese black (Taniguchi et al., 2004). This mutation has been also studied in Italian cattle breeds (Moioli et al., 2007) and other studies also demostrated that *SCD1* polymorphisms contributed to variations in FA composition of Japanese Black cattle (Matsuhashi et al., 2011; Mannen, 2011; Yokota et al., 2012), Holstein (Mele et al., 2007; Narukami et al., 2011), and Korean Hanwoo steers (Lee et al., 2008; Oh et al. 2011; Kim et al., 2017). Similar studies have also been replicated in pork, even lipogenesis is different compared with ruminants, and a mutation positioned in the *SCD* sequence of several putative transcription factor binding sites, was related to an enhanced 9*c*-18:1/18:0 in muscle and subcutaneous fat, but not in liver (Estany et al., 2014). More recently, in river buffalo, the biological role of an SNP in the *SCD* promoter has been also associated with higher MUFA and PUFA content (Gu et al., 2019).

In addition to regulation of FA profile by *SCD1*, a novel Δ9-desaturase isoform *SCD5*, which was previously found in humans, has also been identified in the chromosome 6 of cattle which showed to be highly expressed in brain tissue (Lengi & Corl., 2007). However, *SCD5* has also showed to be expressed in bovine mammary gland (Canovas et

al., 2010). And in a posterior study, it was reported the association between several polymorphisms in *SCD5* and FA composition, showing a SNP (ss252452204) related to an increase in MUFA, 9*c*-14:1 DI and 9*c*,11*t*-CLA content, whereas another SNP (rs43687643) was associated with a decrease in SFA and an increase in PUFA in milk of Holstein-Friesian (Rincon et al., 2012). It thus appears that both bovine isoforms, *SCD1* and *SCD5,* contribute to the FA composition. In beef cattle, the allele g.179412T>G of *SCD5* has been correlated with the 9*c*-16:1 DI in muscle FA composition of Brangus steers, suggesting a putative substrate specificity between SCD isoforms (Baeza et al., 2012). However, the expression of *SCD5* and its relation to the subcutaneous FA profile has not been investigated yet.

II. HYPOTHESIS AND OBJECTIVES



The current Ph.D. Thesis has been conducted within BIOMICs and Lactiker Research Groups at the University of the Basque Country (UPV/EHU), which are dedicated to perform multidisciplinary research on the field of genetics, and quality of foods from animal origin, respectively. Therefore, this thesis has been accomplished through an integrative approach using genetics and fat quality determination tools. In addition, the present project has been developed in close collaboration with local breeder associations and abattoirs.

The present work is based on the following **hypotheses**:

1) A thorough genetic characterization of allochthonous and autochthonous cattle breeds can help to study within breed variability and ensure appropriate diversity parameters to support future breeding programmes. Moreover, these actions can support the maintenance of local animal genetic resources and, overall the Basque agricultural heritage.

2) It is known that the fatty acid profile of beef, as a nutritional quality trait, is dependent on breed and overall management practices. A thorough characterization of the fatty acid composition together with the understanding of the lipogenic gene regulation and lipogenic gene's variability related to the fatty acid profile will help to unravel these associations and, consequently, will aid the quality enhancement of meat and derived products from bovine breeds.

In order to address the proposed hypotheses, the present Ph.D. Thesis is focused in the following **main objectives**:

Objective 1. To perform a genetic characterization of several bovine breeds well established in the Basque region in order to study their genetic diversity and phylogenetic relationship with other bovine breeds from Europe.

In order to achieve this first objective, the following specific objectives were set:

1.1. To study the effectiveness and discrimination power of microsatellite markers in several cattle breeds for parentage and identification purposes.

1.2. To investigate the genetic diversity, founder effect, historical migrations alleles and the phylogenetic relationships between autochthonous and allochthonous cattle breeds.

1.3. To assess the traceability through genetic assignments comparing several microsatellite loci panels using different predictive models.

1.4. To establish the most discriminant markers in breeds raised in the Basque region to be included in a STR panel for an appropriate determination of an unknown cattle individual to its population of origin.

Objective 2. To characterize the fatty acid composition of adipose tissue obtained from commercial bovine carcasses in order to understand and relate this profile to several lipogenic genes in breeds well established in the Basque region.

In order to achieve this second objective, the following specific objectives were set:

2.1. To characterize the fatty acid composition of commercially obtained beef adipose tissue (subcutaneous fat) from a wide range of management practices.

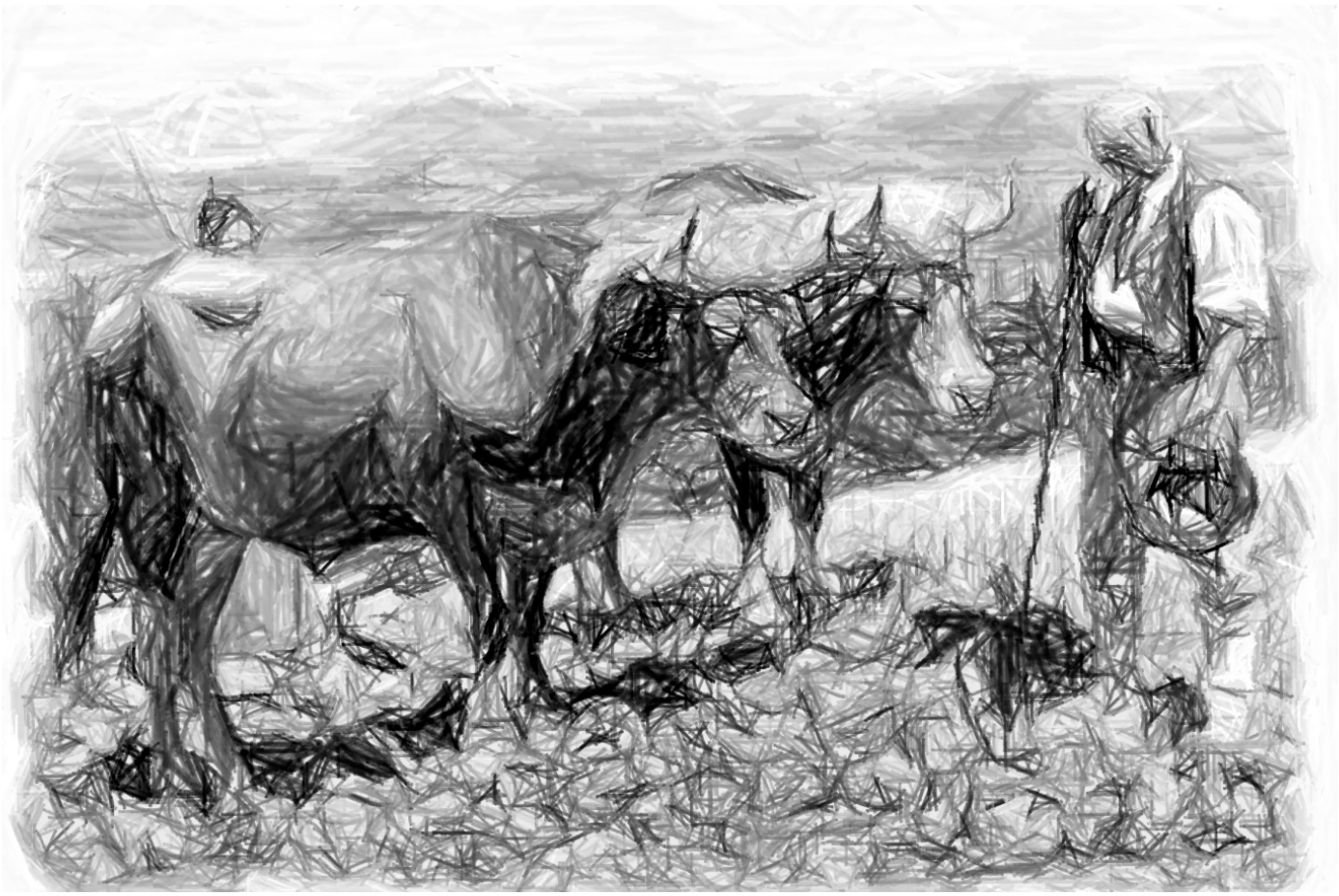
2.2. To analyse the gene expression of several lipogenic genes, and their relationship, of adipose tissue obtained from bovine carcasses.

2.3. To investigate the desaturation indexes, and their correlations with lipogenic gene expression, obtained from substrate and product fatty acids in relation to Δ9-desaturase.

2.4. To identify genetic polymorphisms in lipogenic genes of cattle, analyse variability and study the polymorphism similarity among ruminant and other mammalian species.

2.5. To assess the associations and correlations among genetic polymorphisms, gene expression and fatty acid composition in cattle.

III. MATERIAL AND METHODS



1. EXPERIMENTAL DESIGN AND SAMPLE COLLECTION

The collection of bovine biological samples for the several studies was performed commercially in collaboration with breeder's associations, veterinarians working for the associations, and abattoirs.

1.1. SAMPLING I

Blood samples were collected during an annual diagnostic testing for national cattle disease screening sessions. Blood samples from Terreña (n=28) and Salers (n=403) cattle breeds were collected.

A blood sample per animal was collected from the *coccygeus* (tail) vein using two vacutainer tubes (10 mL) with EDTA as an anticoagulant. The protocol described by ISAG (FAO, 2011) was followed. Blood samples were stored in refrigeration (4 °C) until further analyses.

Results obtained from **sampling I** were published in **Study 1, 2 and 3** corresponding to Publication 1, 2, and 3.

1.2. SAMPLING II

Muscle and adipose tissue samples were obtained over 12 non-consecutive days, and following safety and welfare conditions described by the European Council Regulation (EC) No 1099/2009, from a local commercial abattoir (Urkaiko S. Coop., Zestoa, Gipuzkoa, Spain). Samples from 151 bovine carcasses belonging to four breeds were collected: 114 Pirenaica animals (60 bulls and 54 heifers), 13 Salers bulls and 24 Holstein-Friesian cull cows.

Samples were collected at 24 hours post-mortem. From each carcass approximately 20-30 g of neck (*Sternomandibularis*) muscle and 60-80 g of subcutaneous fat tissues (5-6th ribs of the left half carcass) were sampled as depicted in **Figure III.14.** Muscle samples were transported to the laboratory under refrigeration conditions (4 °C) and stored at -20 °C until further DNA analyses. From each carcass, two samples of adipose tissue were collected for RNA and FA analyses, respectively. First sample was immediately

preserved in cryotubes with RNAlater™ Stabilization Solution (Ambion, Austin, TX, USA) in insulated coolers, transported to the laboratory and stored at -80 °C until further RNA analysis. And an additional 10 g of adipose tissue sample was stored in plastic bags (internal air removed), transported to the laboratory under refrigeration conditions and stored at -80 °C for further FA analysis.

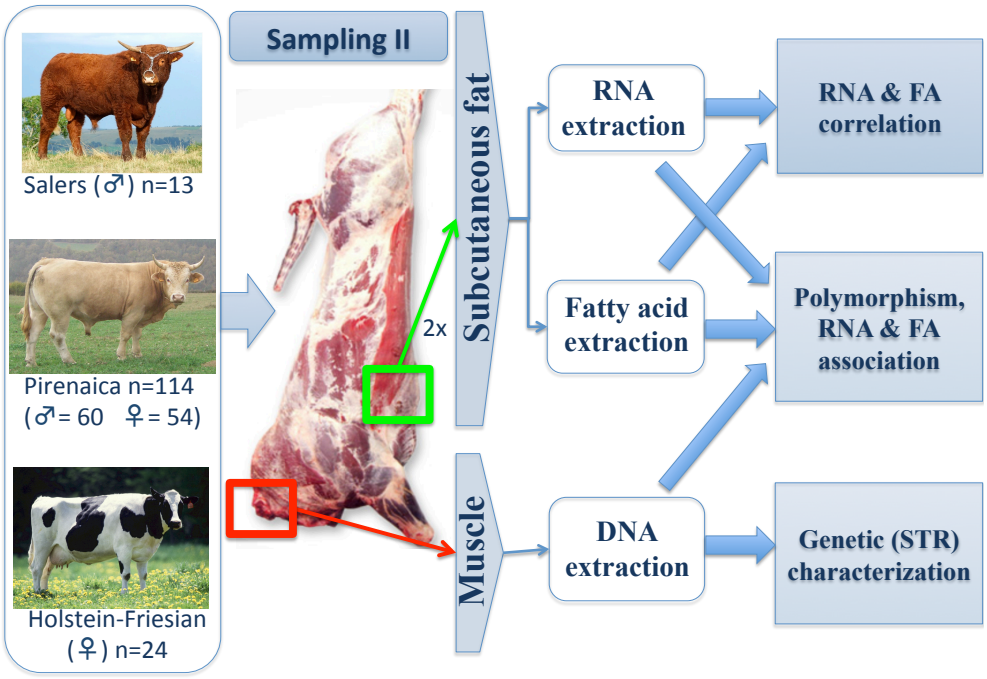


Figure III.14. Schematic description of sampling II and workflow of each study performed.

These samples were destined to (1) the study of lipogenic gene expression, polymorphism variability and its association with fatty acid composition, (2) the study of genetic diversity and phylogeny of cattle, and (3) the assessment of the effectiveness of STR marker sets using new assignment approach by selecting the most polymorphic markers for identification and traceability cases.

Results obtained from **sampling II** were published in **Study 3, 4 and 5** corresponding to Publication 3, 4, and 5.

2. METHODOLOGY

2.1. MATERIALS

2.1.1. LABORATORY EQUIPMENT

- ABI PRISM 3130xl genetic Analyzer, APPLIED BIOSYSTEMS, Wilmington, DE, USA
- ABI PRISM 7500 Sequence Detection System, APPLIED BIOSYSTEMSs, Foster City, CA, USA
- C1000 Touch thermal cycler, BIO-RAD, Hercules, CA, USA
- Centrifuge Allegra X-12 BECKMAN COULTER,Cat No: 41103904 L´Hospitalet de Llobregat, Spain
- Centrigufe Sigma 2-6 (amplificated products), SARTORIUS, Cat No: 10223 Madrid, Spain
- CFX96™ Real-Time PCR Detection System, BIO-RAD, Hercules, CA, USA
- Compact DNA Centrifugal Mini-Evaporator System, DNA mini HETO, Mod. 23900E Madrid, Spain
- DTX 880 Multimode Detector, BECKMAN COULTER, Cat N°: 987921 L´Hospitalet de Llobregat, Spain
- Gel documentation system UVIdoc D55 LCD 20M UVItec, Cat No: U-1642-4542 Cambridge, UK
- GeneAmp® PCR System 9700 Thermal Cycler, APPLIED BIOSYSTEMSs, Foster City, CA, USA
- Laminar flow cabinet, TELSTAR Mod. AV-100 Terrassa, Spain
- Minicentrifuge Microfuge 16, BECKMAN COULTER, Cat No: 270-605133A L´Hospitalet de Llobregat, Spain
- NanoDrop™ ND-1000 Spectrophotometer, THERMOFISHER SCIENTIFIC, Cat No: NC9904842 Waltham, MA, USA
- Roller mixer, LABOLAN, Cat No: C53259, Esparza de Galar, Spain
- Thermomixer® Confort, EPPENDORF, Cat No: 5355000.011 Madrid, Spain
- Unitronic Thermostatic Reciprocating Shaking Bath, GRUPO SELECTA, Cat No: 6032011 Abrera, Spain
- Vortex mixer, VWR, Cat No:444-1372, Linars del Vallés, Spain

2.1.2. REAGENTS

- AmpliTaq Gold® DNA Polymerase THERMOFISHER SCIENTIFIC, Cat No: 4311806, Waltham, MA, USA
- BigDye Terminator v3.1 Sequencing Kit APPLIED BIOSYSTEMS, Cat No: 4337455, Wilmington, DE, USA
- BigDye XTerminator® Purification Kit APPLIED BIOSYSTEMS, Cat No: 4376486, Wilmington, DE, USA
- Bovine Genotypes™ Panel 1.2 THERMOFISHER SCIENTIFICC, Cat No: F-904S, Waltham, MA, USA
- Bovine Genotypes™ Panel 2.2 THERMOFISHER SCIENTIFIC, Cat No: F-847L, Waltham, MA, USA
- Buffer 10X NH_4 without $MgCl_2$ BIOLINE, Cat no: BIO-21040 Barcelona, Spain
- $CHCl_3$ (Chloroform) Sigma-Aldrich Cat no: 288306, m.m. = 119.38, Madrid, Spain
- dNTPs BIOLINE, Cat no: BIO-39028 Barcelona, Spain
- EDTA-Na_2 (Disodium ethylenediaminetetraacetate dihydrate), SIGMA-ALDRICH, Cat No: E-5143, m.m. = 372,24 Madrid, Spain
- Ethanol 100%, SCHARLAB, Cat No: ET0005, m.m. = 46,07, Gentmenat, Spain
- Exonuclease I, 5 U, TAKARA, Cat No: 2650A, St. Germain en Lays, France
- EZ-VISIONTM 6x, AMRESCO LLC, Fountain Parkway, OH, USA
- GelRed Nucleic Acid Gel Stain, BIOTIUM, Fremont, CA, USA
- GeneScan™ – 500LIZ® size standard, APPLIED BIOSYSTEMS, Cat No: 4322682, Wilmington, DE, USA
- Gentra Puregene blood kit, QIAGEN, Cat No: 158389, Qiagen, Valencia, CA, USA
- Hi-Di Formamide™, APPLIED BIOSYSTEMS, Cat No: 4311320 Wilmington, DE, USA
- Isopropanol, SCHARLAB, Cat: AL03111000, SIGMA m.m. = 60,10, Spain
- KCl (Potasium Chroride), PANREAC, Cat No: 131494.1210, m.m. = 74,56 Castellar del Vallés, Spain
- KOD-plus DNA polymerase, TOKOYO, Code No. KOD-201, Osaka, Japan
- $MgCl_2$ (Magnesium Chloride), BIOLINE, Cat no: BIO-21040 Barcelona, Spain
- NaAc (Sodium acetate), MERCK, Cat N°: 106268, m.m. = 82,03 Madrid, Spain
- NaCl (Sodium Chloride), MERCK, Cat N°: 106404, m.m. = 58,44 Madrid, Spain
- NH_4Cl (Amonium Chroride), SIGMA-ALDRICH, Cat N°: A-9434, m.m. = 53,49 Madrid, Spain
- Proteinase K, PROMEGA, Cat No: V3021, Madrid, Spain
- Quant-iTTM PicoGreen ® dsDNA Assay Kit, THERMOFISHER SCIENTIFIC, Cat No: P758,9 Waltham, MA, USA
- ReverTra Ace™ qPCR RT Master Mix, TOYOBO, Code No. FSQ-201, Osaka, Japan

- RNase lipid mini kit, QIAGEN, Cat. No.: 74804, Valencia, CA, USA
- RNase-Free DNase Set, QIAGEN, Cat. No.: Cat. No: 79254, Valencia, CA, USA
- SAP (Shrimp Alkaline Phophatase), 1 U, TAKARA, Cat No: 2660A, St. Germain en Lays, France
- SDS (Sodium dodecil sulfate), SIGMA-ALDRICH, Cat N°: L-4390, m.m. = 288,38 Madrid, Spain
- SsoFast ™ EvaGreen® Supermix, BIO-RAD, Cat No: 30091725202 Alcobendas, Spain

- StockMarks® for Cattle Bovine Genotyping Kit, THERMO SCIENTIFIC, Cat No: 4307480, Waltham, MA, USA
- TaqMan Gene Expression Master Mix, APPLIED BIOSYSTEMS, 4369016, Wilmington, DE, USA

  Tris base (Tris (hidriximetil)-aminometane), AFFYMETRIX, Cat No: 75825, m.m. = 121,14 High Wycombe, UK.

2.2. GENETIC METHODS

2.2.1. EXTRACTION OF NUCLEIC ACIDS

Depending on the type of sample, several commercial kits for nucleic acid extraction were employed. Generally, instructions given in the manual were followed and the reagents included in the kit were used, except for cases where modifications were necessary. These modifications have been described in the corresponding sections.

2.2.1.1. DNA extraction (Sampling I & II)

Two methods were used for DNA extraction depending on sample type:

1) DNA Extraction from whole blood (Sampling I)

This procedure is for extraction and purification of DNA from fresh samples of 20 ml whole blood using the Gentra Puregene Blood Kit (Qiagen, Valencia, CA, USA) and a Homemade Reagents Kit with an optimized protocol. The protocol can also be used for DNA extraction and purification from frozen blood, packed cells, buffy coat or bone marrow:

Centrifuge 2 vacutainers (10 ml each) containing whole blood for 15 min at 1500 *g*, remove the supernatant plasma and transfer the buffy coat containing the white blood

cells (WBCs) and red blood cells (RBCs) to a new falcon tube. Add 7 ml of PBC 1x buffer to falcon tube (with buffy coat) and incubate 1-3 hours at room temperature (RT) in a roller mixer. Add 30 ml of RBC lysis buffer and incubate for 10-30 min in a roller mixer. Centrifuge falcon tube for 5 min at 2000 *g*, eliminate supernatant and vortex the remaining WBCs pellet. Add 10 ml of Cell lysis buffer to the falcon containing WBCs. Incubate at 37 °C until the solution is homogeneous. Samples are stable in this solution 2 years at RT. Vortex 20 s and cool in ice for 4 min the falcon tube with lysed WBCs. Add 3.33 ml of Protein precipitation solution (QIAGEN, CA, USA) and vortex vigorously 20 s. Centrifuge falcon tube during 6 min at 2000 *g*. Prepare a new falcon tube with 10 ml of cold isopropanol and keep in freezer 10 min (-20 °C). Transfer the supernatant from first falcon tube to the tube containing isopropanol. Take care not to dislodged brown protein pellet. Discard protein pellet tube. Invert vigorously the tube containing supernatant and isopropanol during 5 min until DNA precipitation. Centrifuge falcon tube for 3 min and 2000 *g*. Carefully discard supernatant and drain the tube to dry pellet but taking care to keep DNA pellet in the tube. Add 10 ml of 70 % Ethanol, invert tube to wash the DNA pellet and centrifuge for 1 min and 2000 *g*. Carefully discard supernatant inverting the tube or with pipette to dry pellet but taking care not to drop DNA pellet from tube. Air-dry the pellet for 15 min. Add 1 ml of DNA hydration solution or milli-Q water and Incubate 65 °C for 1 hour and leave overnight at RT. Storage at -20 °C or go for DNA quantification and further DNA dilutions.

2) DNA extraction from muscle (Sampling II)

This procedure is for purification of DNA from 0.2 g of muscle per sample:

Add 0.2 g of muscle tissue to a 2 mL Eppendorf tube. Add 588 µl of lysis buffer (TRIS 50mM, EDTA 100mM, NaCl 0.1 mM, SDS 1%) and 12 µl of proteinase K to the Eppendorf tube. Incubate at 55 °C overnight in a vortex mixer. Add Protein Precipitation buffer. Vortex 20 s and centrifuge at 13.000-16.000 rpm for 3 min. Prepare a new Eppendorf tube with 600 µl of cold isopropanol and keep in freezer 10 min (-20 °C). Transfer the supernatant from first Eppendorf tube to the tube containing isopropanol. Take care not to dislodged brown protein pellet. Discard protein pellet tube. Invert vigorously the tube containing supernatant and isopropanol during 5 min until DNA precipitation. Centrifuge Eppendorf tube for 1 min at 13.000-16.000 rpm. Carefully discard supernatant and drain the tube to dry pellet but taking care to keep DNA pellet in the tube. Add 300 µl of 70 % ethanol, invert tube to wash the DNA pellet and

centrifuge for 1 min at 13.000-16.000 rpm. Carefully discard supernatant inverting the tube or with pipette to dry pellet but taking care not to drop DNA pellet from tube. Air-dry the pellet for 15 min. Add 40 µl of DNA hydration solution or milli-Q water and incubate at 65 °C and 900 rpm for 30 min.

2.2.1.2. RNA Extraction (Sampling II)

Total RNA was extracted from adipose tissue conserved in a cryotube with RNAlater® during transport and stored at -80 °C. RNase lipid mini kit (QIAGEN, Valencia, CA, USA) was used following a modified protocol:

RNA extraction:

Prepare a 1.5ml Eppednorf tube with 1 ml of QIAZOL and identify it with each sample code and conservate at 4 °C. Set the centrifuge, that will be used in next steps, at 4 °C. Prepare 100 mg of adipose tissue for cryotube. Mortar and pestle, previously sterilized, were used to pulverize about of each 100 mg sample in liquid nitrogen; samples were crushed until a fine powder was obtained. Approximately 50 to 100 mg powder was recovered from each sample and placed into a cold 1.5 ml Eppendorf tube with QIAZOL. Homogenate, powder with QIAZOL by inverting the tube to help cell lysis from tissue samples. If fat micelles are formed vortex the tube to help RNA extraction. Cultivate at RT (15-25 °C) for 5 min to help to the dissociation of nucleoprotein complexes. Centrifuge at 12000 *g* for 10 min at 4°C. A 3-phase tube is observed: Top phase (fats), middle phase (RNA supernatant) and lower organic phase (extracellular membranes, DNA polysaccharides, etc.). Prepare a new 1.5 ml Eppendorf tube with 200 µl chloroform (Sigma-Aldrich). Using a micropipette, pass through the top phase (fats) until the middle phase containing the colorless RNA supernatant phase and collect it into the tube with 200 µl chloroform. Vigorously hand shaken, and incubated at RT for 2-3 min. Centrifuge this solution for 12,000 *g* for 15 min at 4°C. 3-phase tube is observed, from which the top aqueous phase (RNA phase) will be collected into a new cold 1.5 ml Eppendorf tube. Add 600 µl of cold 70 % Ethanol and vortex for 20-30 s.

RNA purification with QIAGEN spin column:

Set up centrifuge at RT (15–25°C). Transfer a maximum of 700 µl to an RNeasy Mini spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge

for 15 s at 8000 *g* (10,000 rpm) at RT. Discard the flow-through that passed to the bottom of collection tube. Repeat previous step until all the sample (RNA supernatant + ethanol) has been purified through the RNeasy Mini spin column.

NOTE: Eppendorf with flow-through liquid (700 µl + 700 µl) can be conserved to recover miRNAs separately.

On-Column DNase digestion (recommended for RT-PCR):

Add 350 µl of Buffer RW1 to RNeasy Mini spin column and centrifuge 15 s for 8000 *g* (10000rpm) to clean the membrane. Discard bottom flow-through liquid. Prepare DNase I incubation mix by adding 10 µl DNase stock solution to 70 µl of RDD buffer and centrifuge breafly to recover full volume. Add 80 µl of DNase I incubation mix to the RNeasy Mini spin column and incubate at RT for 15 min. Add 350 µl of RW1 buffer to the RNeasy Mini spin column and centrifuge at 8000 *g* (10000 rpm) for 15 min. Discard bottom flow-through liquid.

Only 1-step if On-Column DNase digestion was not used:

Add 700 µl of RW1 to RNeasy Mini spin column and centrifuge at 8000 *g* (10000 rpm) for 15 s. Discard bottom flow-through liquid.

RNA resuspension:

Add 500 µl of RPE buffer to the RNeasy Mini spin column and centrifuge at 8000 *g* (10000 rpm) for 15 s. Discard bottom flow-through liquid. Add 500 µl of RPE buffer to the RNeasy Mini spin column and centrifuge at 8000 *g* (10000 rpm) for 2 min to wash membrane and ensure that long centrifugation has dried the membrane. Discard bottom flow-through liquid. Move the RNeasy Mini spin column to a new 2 ml RNase free Eppendorf tube and centrifuge at full speed for 1 min to eliminate any RPE buffer. Move the RNeasy Mini spin column to a new 1.5 ml RNase free Eppendorf tube. Add 30-50 µl of Free-RNase water and centrifuge at 8000 *g* (10000 rpm) for 1 min. Add extra 30-50 µl of Free-RNase water and centrifuge at 8000 *g* (10000 rpm) for 1 min.

2.2.2. QUANTIFICATION OF NUCLEIC ACIDS

Nucleic acids (DNA and RNA) obtained from sampling I and II were quantified using two methods:

1) Spectrophotometry using a Scientific NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA):

A A_{260}/A_{280} ratio of ~1.8 was generally accepted as "optimal" for DNA and a A_{260}/A_{280} ratio of ~2.0 is generally accepted as "optimal" for RNA. If the ratio was appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. The A_{260}/A_{230} ratio was also used as a secondary measure of nucleic acid purity. Expected A_{260}/A_{230} values were commonly in the range of 2.0-2.2. If the ratio was appreciably lower than expected, it may indicate the presence of contaminants, which absorb near 230 nm such as EDTA, carbohydrates, phenol and TRIzol.

2) Fluorimetry using Quanti-iT PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific, Wilmington, DE, USA):

PicoGreen is a fluorochrome that selectively binds dsDNA and has an excitation maximum at 480 nm (lesser peaks in the short-wave UV range) and an emission peak at 520 nm. PicoGreen have a threshold of <1.0 ng dsDNA per sample, regardless of the detection equipment, or molecular complexity of the sample. When bound to dsDNA, fluorescence enhancement of PicoGreen is exceptionally high and stable to photo-bleaching, whereas little background occurs since the unbound dye has virtually no fluorescence. According to the number of DNA samples, a Master Mix solution was prepared with milli-Q water, PicoGreen dye diluted at 1:200 and TE buffer 20x at 1:20. For standard curve, 4 µl of control DNA (genomic DNA from the eukaryotic λ phage enterobacteria: 100 ng/µl of) was diluted in 196 µl of Master Mix solution. Standard curves were constructed by serial dilution of master mix containing the Control DNA. For measurements, Master Mix solution (99 µl) and 1 µl of sample DNA was prepared. Master Mix and Sample DNA solution was cultivated during 45 min at 37 °C, before fluorescence measurement in a DTX 880 Multimode Detector (Beckman Coulter, CA, USA).

After quantification, the nucleic acids were diluted in milli-Q water to a 100 ng/µl to be stored at −20 °C and aliquots (50 ng/µl) were produced. Aliquots of RNA were stored at

−80 °C and dehydrated in RNAstable 96-Well Plates (Biomatrica, San Diego, CA, USA) for long-term storage.

2.2.3. AMPLIFICATION OF NUCLEIC ACIDS

The general workflow was followed for the analysis of STRs and genetic polymorphisms as described in **Figure III.15**.

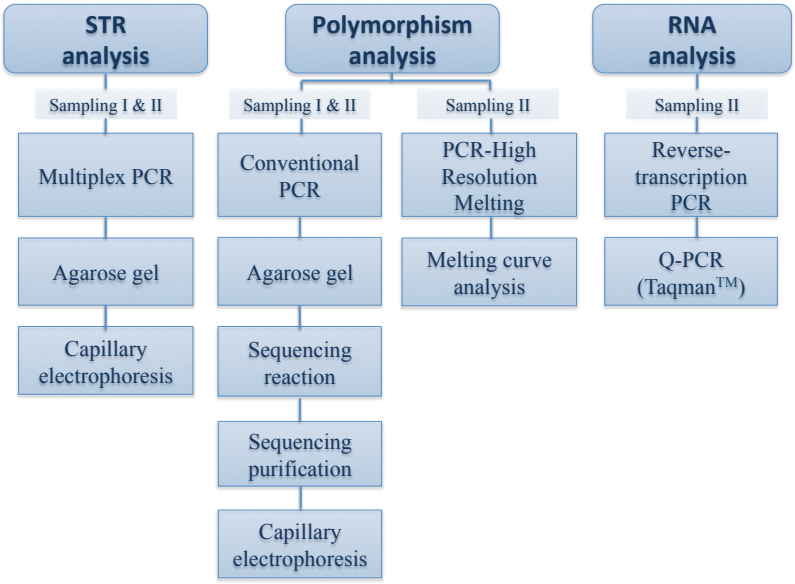


Figure III.15. A general workflow showing different strategies of DNA amplification used. Q-PCR: quantitative PCR.

2.2.3.1. Primer design

PCR amplification primers were designed with the software PerlPrimer v.1.1.21.313 (Marshall, 2004). The specificity of the primers and their non-homology with other genome regions were confirmed with Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Potential unfavorable interactions between primers were checked with the web-based version of AutoDimer 3.14 (Vallone and Butler, 2004) (https://www-s.nist.gov/dnaAnalysis/index.do).

The optimal annealing temperature for each primer was assessed by testing the performance of the primers in temperatures between 55-62 °C.

2.2.3.2. PCR amplification

2.2.3.2.1. Multiplex PCR and capillary electrophoresis

Microsatellite Multiplex PCR and capillary electrophoresis (fragment analysis) was performed following several protocols and depending on the marker set used:

Bovine Genotypes Panel 1.2 Kit (by Thermo Scientific™):

Each sample was amplified in a total volume of 5 µl containing 2 µl of Bovine Genotypes Master Mix (F-841), 2 µl Bovine Genotypes Panel 1.2 Primer Mix (F-906) and 2 ng of DNA sample that was carried out in a GeneAmp® PCR System 9700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with the following conditions: pre-incubation at 98°C for 1 min, 30 cycles (98 °C for 20 s, annealing at 60°C for 75 s, extend 72°C for 30 s) and a final extension at 72 °C for 5 min.

For the capillary electrophoresis called fragment analysis, prepare a mix of 0.3 µl of GeneScan™ 500 LIZ® Size Standard (Cat. Num 4322682; Applied Biosystems, Foster City, CA, USA), 11 µl of formamide and 1-2 µl of amplified multiplex PCR product and run a denaturalization at 95 °C for 5 min and 4 °C for 4 min.

StockMarks® for Cattle Bovine Genotyping Kit (by Thermo Scientific™):

Prepare a total of 15 µl reaction volume in a 0.2 or 0.5 ml PCR tube containing 3 µl of StockMarks® PCR Buffer, 4 µl of dNTP mix, 0.5 µl of AmpliTaq Gold® DNA Polymerase, 5.5 µl of Amplification primer mix, 1 µl milli-Q water and 1 µl of sample DNA (1-10 ng/ µl). Use a GeneAmp® PCR System 9700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with the following conditions: pre-incubation at 95°C for 10 min, 30 cycles (94 °C for 45 s, annealing at 50% ramp 61°C 45 s, extend at 80% ramp 72°C 60 s), a final extension at 72 °C for 60 min and final step at 25 °C for 2 h.

For the capillary electrophoresis called fragment analysis, prepare a mix of 0.5 µl of GeneScan™ 500 LIZ® Size Standard (Cat. Num 4322682; Applied Biosystems, Foster City, CA, USA), 11.5 µl of formamide and 1-2 µl of amplified multiplex PCR product and run a denaturalization at 95 °C for 2 min and 4 °C for 3 min.

30 STR marker panel:

Each sample was amplified using the primers suggested by FAO (2011) as described in **Table III.1.** Five multiplex PCRs were designed to amplify a 30 STRs panel set. Each multiplex PCR was carried out in a final volume of 15 µl containing 0.5 U of AmpliTaq Gold (Applied Biosystems), 1.5 µl of 10X PCR buffer, 0.2 mM dNTP (Takara Biotechnology Co, Ltd, Shiga, Japan), 8 µl of milli-Q water, 100 ng of DNA sample and 2 µl of Primer Mix. Primer Mix was prepared using a dilution 1:300 of each primer (0.15mM) containing FAM (blue), VIC (green), NED (yellow) dyes as described in each multiplex group (**Table III.3.**). Multiplex PCR was carried out in a GeneAmp® PCR System 9700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with the following conditions: pre-incubation at 95°C for 19 min, 40 cycles (95 °C for 30 s, annealing at 55°C for 30 s, extend 72°C for 1min) and a final extension at 72 °C for 7 min.

For the capillary electrophoresis called fragment analysis, prepare a dilution of 5:1000 of GeneScan™ 500 LIZ® Size Standard (Cat. Num 4322682; Applied Biosystems, Foster City, CA, USA). Add 10 µl of formamide and 1-2 µl of amplified multiplex PCR product and run a denaturalization at 95 °C for 5 min and 4 °C for 4 min.

Table III.3. The multiplex groups (5 PCR reactions), STR markers and labels (**FAM**, **HEX** or NED).

Marker name(s)	Ch	primer sequence (5′ - 3′)	Temp (°C)	Genebank accession number	Allele range (bp)	Gr.	Dye
INRA063 (D18S5)	18	F ATTTGCACAAGCTAAATCTAACC R AAACCACAGAAATGCTTGGAAG	55-58	X71507	167-189	1	HEX
INRA005 (D12S4)	12	F CAATCTGCATGAAGTATAAATAT R CTTCAGGCATACCCTACACC	55	X63793	135-149	1	FAM
ILSTS005 (D10S25)	10	F GGAAGCAATGAAATCTATAGCC R TGTTCTGTGAGTTTGTAAGC	54-58	L23481	176-194	4	FAM
HEL5 (D21S15)	21	F GCAGGATCACTTGTTAGGGA R AGACGTTAGTGTACATTAAC	52-57	X65204	145-171	3	FAM
HEL1 (D15S10)	15	F CAACAGCTATTTAACAAGGA R AGGCTACAGTCCATGGGATT	54-57	X65202	99-119	1	HEX
INRA035 (D16S11)	16	F TTGTGCTTTATGACACTATCCG R ATCCTTTGCAGCCTCCACATTG	55-60	X68049	100-124	4	FAM
ETH152 (D5S1)	5	F TACTCGTAGGGCAGGCTGCCTG R GAGACCTCAGGGTTGGTGATCAG	55-60	Z14040	181-211	2	NED
ETH10 (D5S3)	5	F GTTCAGGACTGGCCCTGCTAACA R CCTCCAGCCCACTTTCTCTTCTC	55-65	Z22739	207-231	5	FAM
HEL9 (D8S4)	8	F CCCATTCAGTCTTCAGAGGT R CACATCCATGTTCTCACCAC	52-57	X65214	141-173	2	NED

CSSM66 (D14S31)	14	F	ACACAAATCCTTTCTGCCAGCTGA	55-65	...	171-209	1	FAM
		R	AATTTAATGCACTGAGGAGCTTGG					
INRA032 (D11S9)	11	F	AAACTGTATTCTCTAATAGCTAC	55-58	X67823	160-204	3	NED
		R	GCAAGACATATCTCCATTCCTTT					
ETH3 (D19S2)	19	F	GAACCTGCCTCTCCTGCATTGG	55-65	Z22744	103-133	2	FAM
		R	ACTCTGCCTGTGGCCAAGTAGG					
BM2113 (D2S26)	2	F	GCTGCCTTCTACCAAATACCC	55-60	M97162	122-156	5	FAM
		R	CTTCCTGAGAGAAGCAACACC					
BM1824 (D1S34)	1	F	GAGCAAGGTGTTTTTCCAATC	55-60	G18394	176-197	4	HEX
		R	CATTCTCCAACTGCTTCCTTG					
HEL13 (D11S15)	11	F	TAAGGACTTGAGATAAGGAG	52-57	X65207	178-200	3	FAM
		R	CCATCTACCTCCATCTTAAC					
INRA037 (D10S12)	10	F	GATCCTGCTTATATTTAACCAC	57-58	X71551	112-148	1	NED
		R	AAAATTCCATGGAGAGAGAAAC					
BM1818 (D23S21)	23	F	AGCTGGGAATATAACCAAAGG	56-60	G18391	248-278	2	HEX
		R	AGTGCTTTCAAGGTCCATGC					
ILSTS006 (D7S8)	7	F	TGTCTGTATTTCTGCTGTGG	55	L23482	277-309	2	FAM
		R	ACACGGAAGCGATCTAAACG					
MM12 (D9S20)	9	F	CAAGACAGGTGTTTCAATCT	50-55	Z30343	101-145	3	NED
		R	ATCGACTCTGGGGATGATGT					
CSRM60 (D10S5)	10	F	AAGATGTGATCCAAGAGAGAGGCA	55-65	...	79-115	1	FAM
		R	AGGACCAGATCGTGAAAGGCATAG					
ETH185 (D17S1)	17	F	TGCATGGACAGAGCAGCCTGGC	58-67	Z14042	214-246	3	NED
		R	GCACCCCAACGAAAGCTCCCAG					
HAUT24 (D22S26)	22	F	CTCTCTGCCTTTGTCCCTGT	52-55	X89250	104-158	4	HEX
		R	AATACACTTTAGGAGAAAAATA					
HAUT27 (D26S21)	26	F	AACTGCTGAAATCTCCATCTTA	57	X89252	120-158	2	HEX
		R	TTTTATGTTCATTTTTTGACTGG					
TGLA227 (D18S1)	18	F	CGAATTCCAAATCTGTTAATTTGCT	55-5	…	75-105	5	FAM
		R	ACAGACAGAAACTCAATGAAAGCA					
TGLA126 (D20S1)	20	F	CTAATTTAGAATGAGAGAGGCTTCT	55-58	…	115-131	5	NED
		R	TTGGTCTCTATTCTCTGAATATTCC					
TGLA53 (D16S3)	16	F	GCTTTCAGAAATAGTTTGCATTCA	55	…	143-191	3	HEX
		R	ATCTTCACATGATATTACAGCAGA					
SPS115 (D15)	15	F	AAAGTGACACAACAGCTTCTCCAG	55-60	FJ828564	234-258	5	HEX
		R	AACGAGTGTCCTAGTTTGGCTGTG					
ETH225 (D9S1)	9	F	GATCACCTTGCCACTATTTCCT	55-65	Z14043	131-159	5	HEX
		R	ACATGACAGCCAGCTGCTACT					
TGLA122 (D21S6)	21	F	CCCTCCTCCAGGTAAATCAGC	55-58	…	136-184	4	NED
		R	AATCACATGGCAAATAAGTACATAC					
INRA023 (D3S10)	3	F	GAGTAGAGCTACAAGATAAACTTC	55	X67830	195-225	4	NED
		R	TAACTACAGGGTGTTAGATGAACTC					

Ch: Chromosome. Gr.: Multiplex Group. Temp: Annealing temperature. F: Forward. R: Reverse.

2.2.3.2.2 Conventional PCR

Conventional PCR amplification was performed for the amplification of lipogenic gene's regions of interest. The PCR amplifications were carried out in a final volume of 25 µl containing 0.5 U of KOD-plus DNA polymerase (TOYOBO), 2.5 µl of 10X PCR buffer, 1 mM MgSO4, 25–50 ng of genomic DNA, 0.2 mM dNTP (Takara Biotechnology Co, Ltd, Shiga, Japan), and 0.4 µM of each primer.

The amplifications were carried out on the GeneAmp® PCR System 9700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) which consisted in amplification conditions as follows: pre-incubation at 94°C for 2 min, 30 cycles (94°C for 5 s, annealing at 60°C for 15 s, 68°C for 1.5 min) and a final extension at 72°C for 10 min.

2.2.3.3. Agarose gel electrophoresis

After PCR reaction, agarose gel electrophoresis was used to confirm the DNA Amplification. PCR products were migrated in 1.5% agarose gels in TBE 1x solution and one of the following UV fluorescent dyes:

a) 1:1 GelRed (Biotium, Fremont, CA, USA) and 1.5 µl of PCR product at at 100 V for 30 minutes.

b) 1:6 EZ-VISION™ 6x (AMRESCO LLC, Fountain Parkway, OH, USA) and 5 µl of PCR product at 100 V for 20 minutes.

Agarose gel was observed in an UVIdoc gel documentation system (Uvitec, Cambridge, UK).

2.2.3.4. Purification and sequencing reaction of amplified DNA

Purification:

Nucleotide and primer excess from the PCR amplification were cleaned enzymatically by adding 2 µL of ExoSAP-IT™ (Life Tecnologies, Carlsbad, CA, USA) to 5 µl of PCR product and incubated for 45 min at 37 °C followed by 15 min at 80 °C.

Sequencing Reaction:

The sequencing reactions were carried out using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Wilmington, DE, USA) under the following conditions: 1 µl of Milli-Q water, 6 µl of Buffer 2.5x, 2 µl of BDT, 4 µl of the corresponding primer at 10 µM and 2 µl of PCR product in a final volume of 15 µl. The sequencing reaction consisted on 10 min at 96 °C, 25 cycles at 96 °C for 10 s, 5 s at 55 °C and 4 min at 60 °C in a GeneAmp® PCR System 9700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA).

2.2.3.5. Sequencing product purification

In order to eliminate the unincorporated BigDye terminators and salts, two different purification protocols were used depending on the PCR product:

a) BigDye Xterminator^{TM} Purification Kit (Thermo Fisher Scientific, Wilmington, DE, USA) in the following conditions: 22.5 µl of SAM solution, 5 µl of Xterminator solution and 10 µl of sequencing product. The samples were strong vortexed for 45 min at 2,000 rpm in darkness and then centrifuged for 4 min at 2,000 rpm.

b) Sephadex G-50 fine (e.g. Amersham Pharmacia 17-0042-01 100 gm) was used in following conditions: Using a Millipore MultiScreen-HV Filter Plate (0.45 µm; Cat. Num. MAHVN4550), fill ½ of each well with Sephadex G-50 and 300 µl of milli-Q H_2O, cultivate for 2 hours at RT or overnight. Centrifuge 2200 rpm (900 *g*) for 5 min with a collection plate under Millipore plate to remove excess of water. Add Big Dye sequencing PCR product to Millipore plate wells containing Sephadex. Install ABI sequencing plate under Millipore plate and centrifuge for 5 min at 900 *g*.

2.2.3.6. Capillary electrophoresis and data analysis

Sequencing products were analyzed by mixing 5 µl of Hi-Di™ Formamide (Thermo Fisher Scientific, Wilmington, DE, USA) and 5 µl of purified product. Capillary electrophoresis was conducted on an ABI PRISM 3130 genetic Analyzer and ABI PRISM 3130xl genetic Analyzer (Thermo Fisher Scientific, Wilmington, DE, USA) with POP-7® polymer and a capillary of 36 cm. The results were analyzed using the software Sequencing Analysis v.5.2 (Thermo Fisher Scientific, Wilmington, DE, USA),

ChromasPro v.1.5 (Technelysium Pty Ltd, Brisbane, Australia) and Unipro UGENE v31 (Okonechnikov et al., 2012).

2.2.4. HIGH RESOLUTION MELTING (HRM)

High Resolution Melting (HRM) was optimized for a fast, simple and highly reliable genotyping method. The genotypes of an Indel polymorphism were previously confirmed by simple sequence length polymorphism (SSLP) of PCR amplicons or Sanger sequencing.

The HRM reaction contained 2.5 µl of SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA), 0.75 µl of each primer at 1 µM and 1 ng of DNA in a final volume of 5 µl, and was carried out in a C1000^{TM} Thermal Cycler (Bio-Rad, Hercules, CA, USA) equipped with a CFX96^{TM} Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The HRM conditions were performed following an initial denaturation at 2 min at 98 °C; 40 cycles at 98 °C for 30 s, 30 s at 50 °C of annealing temperature. Plate reads were taken after the initial step of 30 s at 95 °C and 2 min at 60°C, and the melting curve went from 65°C to 95°C with an increment of 0.5°C every 5 s.

Data interpretation was carried out using the software Precision Melt Analysis v.1.2 (Bio-Rad, Hercules, CA, USA). Only high-quality amplification and melting curves with a cluster assignment over 95% of confidence were considered. Three genotypes confirmed by Sanger sequencing, were used for performing the assignment of the corresponding allelic variants of every cluster.

2.2.5. GENE EXPRESSION

2.2.5.1 Reverse transcription

Reverse transcription was performed in a 20 µL final reaction volume containing 220 ng total RNA, 4 µL of 5x RT buffer, 2 µL dNTPs, 1 µL RNAase inhibitor, 1 µL random primer, and 1 µL high efficient ReverTra Ace reverse transcriptase (TOYOBO, Osaka, Japan). The amplifications were carried out on the GeneAmp® PCR System 9700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) which consisted in amplification conditions as follows: 30 °C for 10 min, annealing at 42 °C for 20 min, 99 °C for 5 min, 4 °C for 5 min.

2.2.5.2 Quantitative PCR (Q-PCR)

Custom TaqMan Assays (Applied Biosystems, Foster City, CA, USA) were conducted to measure the relative expression levels of bovine genes using the primers and FAM/TAMRA probes reported in **Table III.3**. Each candidate gene was amplified in multiplex with an internal control (18S rRNA Endogenous Control VIC/TAMRA Probe, Primer Limited) by the co-application reverse transcription method (Co-RT) (Zhu et al., 2005). This multiplexing approach guarantees the same conditions (thus equal amplification efficiency) and same reverse transcriptase activity for both genes, thereby yielding better normalization and reproducibility.

Table III.4. Primer sequences, product sizes and annealing temperatures of bovine genes analyzed by Q-PCR.

Gene [GenBank accession]	Primer sequence (5′ - 3′)	Product (bp)	Annealing T
***SCD*1** **[NM_173959]**	P: CCTCTGGAACATCACCAGCTTCTCGGC F: GCTGTCAAAGAAAAGGGTTCCAC R: AGCACAACAACAGGACACCAG	106	60
***SCD*5** **[NM_001076945]**	P: CAGAACCCGCTCGTCACCCTGGG F: CCCTATGACAAGCACATCAGCC R: GATGGTAGTTATGGAAACCTTCACC	82	60
***SREBP*1** **[NM_001113302]**	P: CAGCCCCAGTCCTGGATCAGCCGA F: CTTGGAGCGAGCACTGAATTG R: GGGCATCTGAGAACTCCTTGTC	83	60

P: probe; F: forward; R: reverse; T: temperature.

The reaction mixture included primers (10 μM each), FAM-labeled probe (10 μM), 0.6 μL of 18S RNA Endogenous Standard containing VIC-labeled probe and limited primers, and 2 × TaqMan Gene Expression Master Mix (7.5 μL) (Applied Biosystems). Real-time PCR was performed in triplicate using the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with a standard two-step cycling program of 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The average of the gene expression levels was used for further analyses.

2.3. TOTAL FATTY ACID COMPOSITION

2.2.1. DERIVATIZATION (BASE CATALYZED METHYLATIONS) OF TOTAL FATTY ACIDS

Subcutaneous adipose tissue samples were weighted (50 ± 1 mg), freeze-dried and derivatized using a base catalyzed direct transesterification (2 mL of 0.5 N methanolic base, Supelco) (Kramer et al., 1997). Prior to methylation, 1 mL of methyl tricosanoate (4 mg of 23:0/mL of toluene; Chromasolv for HPLC, > 99.9 %; Sigma-Aldrich) was added as internal standard.

2.2.2. TOTAL FATTY ACID METHYL ESTER FRACTIONATION

Silver-ion solid phase extraction (Ag^+-SPE) technique was utilized to fractionate FA methyl esters (FAME) by differences in the number and/or geometric configuration of double bonds in order to ease the identification of several overlapping peaks. Ag^+-SPE cartridges available from Sigma-Aldrich (Supelco Discovery, 750 mg/6 mL, 54225-U) were used. After conditioning with 4 mL of acetone followed by 4 mL of hexane (Supelco Technical Report, 2006), about 1 mg of total FAME was loaded in the cartridges and 6 mL of the following eight solvents or solvent mixtures (v/v) were added in consecutive order (Kramer et al., 2008) to collect separate FAME fractions. All solvents used were of HPLC grade (hexane, Chromasolv for HPLC, > 97.0 %; acetone, Chromasolv for HPLC, > 99.8 %; and acetonitrile, Chromasolv Plus for HPLC, > 99.9 %; Sigma-Aldrich): (1) hexane:acetone (99:1) to obtain SFA and BCFA; (2) hexane:acetone (96:4) to obtain trans-monounsaturated FA (MUFA); (3) hexane:acetone (90:10) to obtain cis-MUFA plus c,t-CLA, (4) acetone (0:100) to obtain dienes, (5) acetone:acetonitrile (97:3) to obtain trienes, (6) acetonitrile:acetone (94:6) to obtain tetraenes, (7) acetonitrile:acetone (80:20) to obtain pentaenes, and (8) acetonitrile (0:100) to obtain hexaenes. Individually collected FAME fractions were reconstituted in adequate volume of hexane and stored at -80 °C for further gas chromatographic analysis.

2.2.3. CHROMATOGRAPHIC ANALYSIS

2.2.3.1. Reference standards

For FAME identification purposes, several reference standards were used. From Nu-Chek Prep Inc. (Elysian, MN, USA), individual FAMEs (13:0, 19:0, 21:0, 23:0, 26:0, 28:0) and mixtures such as #463 and #603, and #UC-59M CLA were obtained. From Larodan Fine Chemicals (Malmö, Sweden), 20:3n-9 FAME was obtained. From Sigma-Aldrich, Supelco 37 Component FAME Mix (CRM47885) was obtained. From Matreya (Pleasant Gap, PA, USA), the bacterial FAME mixture was purchased.

2.2.3.2. Gas chromatography – flame ionization detection

Gas chromatography – flame ionization detector (GC-FID; Agilent Technologies Model 7890A, Wilmington, DE, USA) coupled with an automatic injector (Agilent Technologies, Model 7693) was used to analyze FAMEs and isolated FAME fractions. Two capillary GC columns were used:

(1) A Supelco SP-2560 column (100 m, 0.25 mm i.d., and 0.20 μm film thickness; Bellefonte, PA, USA) operated at two separate but complementary temperature programs going from 45 °C to 215 °C and plateauing at 175 °C and 150 °C, respectively (Kramer et al., 2008). The total GC-FID run for the 175 program was 86 min and for the 150 program was 110 min.

(2) A SLB- IL111 ionic liquid column (100 m, 0.25 mm i.d., and 0.20 μm film thickness) operated at a temperature program from 168 °C to 185 °C (Delmonte et al., 2011). This second column was used to resolve CLA isomers and improve separation of other co-eluting peaks. The total GC-FID run was of 77.8 min.

Samples were most of the times injected using a 100:1 split ratio but this ratio was modified when needed. In all cases, hydrogen (99.999 % purity, Air Liquide, Madrid, Spain) was used as carrier gas at a constant flow rate of 1 mL/min, and injector and detector ports were set at 250 °C.

2.2.3.3. Identification and quantification of fatty acids methyl esters

The identification of FAMEs was confirmed by commercial standards, FAME fractions performed by Ag^{+}-SPE (Kramer et al., 2008), and by retention times and elution orders reported in the literature of ruminant products (Destaillats et al., 2000; Cruz-Hernandez et al., 2004, 2006; Santercole et al., 2007; Kramer et al., 2008; Alves, & Bessa, 2009, 2014; Gómez-Cortés et al., 2009; Delmonte et al., 2011, 2012).

For quantification purposes, chromatographic peak areas were corrected according to published theoretical response factors (Wolff et al., 1995), and thereafter the absolute contents (mg) of FAME were calculated according to the internal standard. Subcutaneous fat FAME contents were expressed as mg/g of fat and/or as percentages (% of total quantified FAMEs).

2.3. DATA TREATMENT AND STATISTICAL ANALYSIS

2.3.1. PARAMETERS FOR GENETIC DIVERSITY, TRACEABILITY AND PARENTAGE

2.3.1.1. Diversity parameters

GENEPOP 4.2 (Raymond & Rousset, 1995) was used to test for deviations from Hardy-Weinberg equilibrium (HW) using exact test of Guo and Thompson (1992). The probability test used a Markov Chain Monte Carlo method (dememorization 5000, batches 100, iterations per batch 1000). Significant levels were calculated per locus and per breed.

Number of alleles per locus (Na) was calculated by direct count, whereas allelic richness (*Rs*) and estimators of F-statistics (Weir & Cockerham, 1984) were computed using FSTAT 2.9.3 (Goudet, 1995) as follows:

$$Rs = \sum \left[1 - \frac{\binom{2N - N_i}{2n}}{\binom{2N}{2n}} \right]$$

where N_i is the number of alleles of type i among the 2N genes.

Weir and Cockerham (1984) estimators of F-statistics:

$$F = \frac{\sigma_a^2 + \sigma_b^2}{\sigma_a^2 + \sigma_b^2 + \sigma_w^2}$$

$$\theta = \frac{\sigma_a^2}{\sigma_a^2 + \sigma_b^2 + \sigma_w^2}$$

$$f = \frac{\sigma_b^2}{\sigma_b^2 + \sigma_w^2}$$

where σ is the observed component of variance: a for between populations, b for between individuals within populations and w for alleles within individuals. These parameters are also related to Wright´s F-statistics (Wright, 1951) as:

$$F = Wright´s\ F_{IT},\ \theta = Wright´s\ F_{ST} \text{ and } f = Wright´s\ F_{IS}$$

Observed heterozygosities (*Ho*) were calculated by direct count as the proportion of heterozygous individuals observed in a sample of the population, whereas the Expected heterozygosities (*He*) was computed as the probability that two alleles taken at random from the population are different and given by the equation $2\ pq$ (Nei, 1973) using CERVUS 3.0 (Kalinowski et al., 2007):

$$He = 1 - \sum_{i=1}^{k} x_i^2$$

where x_i: frequency of allele i and k: number of alleles.

Polymorphic information content (*PIC*) was computed in Powerstats 1.2 (Promega, Madison, WI, USA) as an indicator to describe genotypic variation of a marker that depends on the number of alleles and the distribution of frequencies (Botstein et al., 1980):

$$PIC = 1 - (\sum_{i=1}^{k} x_i^2) - \sum_{i=1}^{k} \sum_{j=i+1}^{k} 2x_i^2\, x_j^2$$

where k is the number of alleles, x_i, x_j: frequency of alleles i and j respectively. In codominant multiallelic markers, such as STRs, with *PIC* values above 0.5 are considered very informative, those with values between 0.25 and 0.5 moderately informative and those that show values less than 0.25 little informative (Botstein et al., 1980).

The existence of a possible founder effect was studied by determining a significant number of loci with heterozygosity excess, using the software BOTTLENECK (Piry et al., 1999). Sign test and Wilcoxon's sign rank test (Cornuet & Luikart, 1996) were performed under stepwise mutation model (SMM) and two-phase model (TPM; 95% SMM with 5% multi-step mutations and a variance among multiple steps of 12), which is considered best for microsatellite data (Di Rienzo et al., 1994; Piry et al., 1999). In addition, a qualitative descriptor of the allele frequency distribution (mode-shift indicator), which discriminates bottlenecked populations from stable populations, was used (Luikart et al., 1998).

2.3.1.2. Population differentiation

The pairwise genetic distances based on F_{ST} described by Reynolds et al. (1983) for short divergence times, and Slatkin, (1995) were computed and the corresponding significance p values between pairs of the analyzed populations were calculated with Arlequin v.3.5 (10,000 permutations) (Excoffier & Lischer, 2010) as follows:

- Reynolds' distance:

$$D_R = -\log(1 - F_{ST})$$

- Slatkin's linearized F_{ST}'s:

$$D = \frac{F_{ST}}{1 - F_{ST}}$$

This method is based on estimating the proportion of genetic variation found within and between populations, since it allows quantitatively comparing the difference between different populations. The significant p values were adjusted with the sequential Bonferroni correction (α) in order to account for potential Type I errors due to the multiple comparisons performed. Neighbor-joining (NJ) trees (Saitou & Nei, 1987) were constructed to quantify the degree to which bifurcating trees distort genetic relationships between populations using the genetic distances and the TreeFit software (Kalinowski, 2009).

The structure of populations was tested with a Bayesian model method in structure 2.3.4 software (Pritchard et al., 2000) under an admixture model for clusters (K) and using 10 Markov Chain Monte Carlo simulations consisting of 1×10^5 iterations after a burn-in of

5×10^5 iterations. The optimal value of K was selected following the clustering mode described by Kopelman et al. (2015) and the approach ΔK of Evanno et al. (2005) and the web interface Structure Harvester (Earl & VonHoldt, 2012). The genetic structure of the different populations was also studied by factorial correspondence analysis (FCA) with Genetix v.4.05.2 (Belkhir et al., 2004).

In order to quantify patterns of population structure principal component analysis (PCA) was carried out with the software IBM SPSS Statistics v.22 (IBM Corporation, Armonk, NY, USA). A global structure implies that each sampling location is genetically closer to its neighbours than randomly chosen locations, as occurs with spatial groups, clines or intermediate states. In contrast, a stronger genetic differentiation among neighbours than among random pairs of populations characterizes a local structure.

2.3.1.3. Parentage and identification parameters

For individual identification and traceability, power of discrimination (PD), paternity index (PI) and matching probability (MP) were computed using Powerstats 1.2 (Promega, Madison, WI, USA). MP was calculated as the probability to have a match between two individuals sharing an identical genotype profile and chosen at random (Weir, 1996) and the combined matching probability (CMP) was computed with the formula:

$$CMP = (MP_1)(MP_2)\dots(MP_k)$$

which is the overall MP including *k* number of loci.

For parentage purposes, the power of exclusion for each locus (PE) was computed considering the formula: PE = 1 − NE-P in which NE-P is the Non-Exclusion Probability for each microsatellite marker calculated by CERVUS 3.0. PE was calculated in the absence of genetic information from one parent (PE-1P) and power of exclusion when genetic information of both parents was available (PE-2P) and power of exclusion for identity of two siblings (PE-SI). Finally, combined power of exclusion (CPE) was calculated as follows:

$$CPE = 1 - (1 - PE_1)(1 - PE_2)\dots(1 - PE_k)$$

which is the overall PE including *k* number of loci (Jamieson & Taylor, 1997). CPE was calculated for PE-1P (CPE_1), PE-2P (CPE_2) and PE-SI (CPE_{SI}) using different commercial marker panels.

2.3.1.4. Population assignments test

First of all, the assignment of individuals to their breeds was tested using the frequency-based (Rannala, & Mountain, 1997) and the Bayesian-based (Paetkau et al., 1995) methods implemented in Geneclass 2 (Piry et al., 2004). The Bayesian method was computed by simulating 1000 genotypes (using allele frequencies) and a fixed threshold of 0.001. Thus, an individual was considered as correctly assigned to a population when it was excluded from all of the non-origin populations ($p \leq 0.001$), but not from the true population of origin.

On the other hand, a supervised machine-learning approach was used to estimate the mean and variance of assignment accuracy by a Monte-Carlo resampling (100 iterations) cross-validation procedure using r software and AssignPOP package (Chen et al., 2018). Analyses were adjusted by the proportion of individuals and by the STRs with the highest F_{ST} to estimate the minimal number of markers for an accurate assignment. This approach creates randomly selected, independent training and test data panels which avoids introducing high-grading bias (Anderson, 2010), while the proportion of individuals from each source population randomly allocated to the baseline data panel was adjusted to avoid biases associated with unbalanced population sizes (Wang, 2017).

2.3.2. GENE EXPRESION DATA ANALYSIS

Gene expression raw data was obtained from the ABI Prism 7500 SDS software v1.4 and PCR efficiency was monitored by the increase in absolute fluorescence (Ramarkers et al., 2003), mainly because this allows PCR efficiency calculation for individual samples/reactions and prevents problems arising from the use of standard curves. Raw data was exported in Rn format, and imported to LinRegPCR (Heart Failure Research Center, Amsterdam, The Netherlands). LinRegPCR determines baseline fluorescence, sets a window of linearity for each amplicon, and calculates the PCR efficiency (E) per sample and amplicons using an iterative algorithm. Efficiencies were over 90 % for all samples and correlation coefficients were higher than 0.99.

From each sample, the Ct value (cycle threshold) was used as the measurable number of Q-PCR cycles from where the fluorescence signal crosses the base (threshold) line to calculate the comparative threshold cycle method (ΔCt) based on the following formula:

$$\Delta Ct = \mathrm{Ct}_{\,target\ gene} - \mathrm{Ct}_{18s\ rRNA\ gene}$$

Linear regression analyses were conducted to examine the associations between gene expression (ΔCt) of lipogenic genes following a linear regression model:

$$Y_i = \beta_0 + \beta_1 \chi_{i1} + e_i$$

where Y_i is the ΔCt of target gene a; β_0 is overall intercept; β_1 is slope coefficient; X_{i1} is ΔCt of target gene b; e_i is random error component.

2.3.3. TOTAL FATTY ACID DATA ANALYSIS

Statistical analysis was conducted using IBM SPSS Statistics 22.0 for windows (SPSS Inc., IBM Corporation, New York, USA). After checking data for normality and homocedasticity, subcutaneous FAME data was statistically analysed.

FAMEs that are substrate and product of SCD enzyme were used to calculate the desaturation index (DI) by the following formula:

$$Desaturation\ index\ (DI) = \frac{[Product]}{([Substrate] + [Product])}$$

where [Substrate] is the concentration of a FAME (mg / g fat) used as a substrate for an enzymatic reaction and [Product] is the concentration of a FAME (mg / g fat) produced as a result of the same enzymatic reaction.

Then, the following general linear model was used for analysis of variance (ANOVA) and to investigate differences in the FAME composition of subcutaneous fat samples:

$$Y_{ijk} = \mu + CT_i + A_j + HCW_k + e_{ijk}$$

where CT was the commercial type (Salers bulls, Pirenaica bulls, Pirenaica heifers, Holstein-Friesian cows) as fixed effect. Age at slaughter (A) and hot carcass weight (HCW) were included as covariates. The effect of sire was also checked but not included in the model as it was statistically not significant. LSD post hoc test was applied for multiple comparison of means among commercial breeds studied. Significance was declared at $p \leq 0.05$.

2.3.4. RELATIONSHIP BETWEEN GENETICS AND FATTY ACID COMPOSITION DATA

In Study IV, to investigate the relationship between gene expression (ΔCt) and DI, partial Pearson correlations coefficients were used as a measure of the strength and direction of a linear relationship between these two continuous variables while controlling the effect of one or more other continuous variables (also known as 'covariates' or 'control' variables) using IBM SPSS Statistics 22.0 for windows (SPSS Inc., IBM Corporation, New York, USA). Partial Pearson correlations were adjusted for age at slaughter (A) and hot carcass weight (HCW) and significant correlations were declared at $p \leq 0.05$.

In Study V, a general linear model was used for the analysis of variance (ANOVA) to study the recessive model (SS/SL and LL) of *SREBP1* genotype (84 bp-indel) and FA associations in overall population:

$$Y_{ijk} = \mu + IP_i + GE_j + e_{ijk}$$

where Y_{ijk} = dependent variables (FAs); μ = mean value; IP_i = effect of 84 bp-indel polymorphism of *SREBP1* (SS: Homozygote, small type with 84 bp deletion; LL: Homozygote, long type with 84 bp insertion; SL: Heterozygote with 84 bp deletion and insertion); GE_j = covariate, gene expression of *SREBP1* (ΔCt); e_{ijk} = random error component. Fixed effects of age at slaughter, hot carcass weight, fat cover, and effect of sire were also checked but not included in the model ($p > 0.05$). Least significant difference (LSD) post hoc test was applied for multiple comparison of means among genotypes studied considering 84 bp-indel frequencies.

Different general linear models were used to study the additive model (SS vs. SL vs. LL) of *SREBP1* genotype (84 bp-indel) and FA associations in (a) overall population excluding Holstein-Friesian or (b) Pirenaica population only:

$$\text{(a)}\quad Y_{ijk} = \mu + IP_i + S_j + B_k + e_{ijk}$$

$$\text{(b)}\quad Y_{ij} = \mu + IP_i + S_j + e_{ij}$$

where Y_{ijk} or Y_{ij} = dependent variables (FAs); μ = mean value; IP_i = effect of 84 bp-indel polymorphism of *SREBP1* (SS: Homozygote, small type with 84 bp deletion; LL: Homozygote, long type with 84 bp insertion; SL: Heterozygote with 84 bp deletion and

insertion); S_j =covariate, sex; B_k = covariate, breed; e_{ijk} or e_{ij} = random error component. LSD post hoc test were applied.

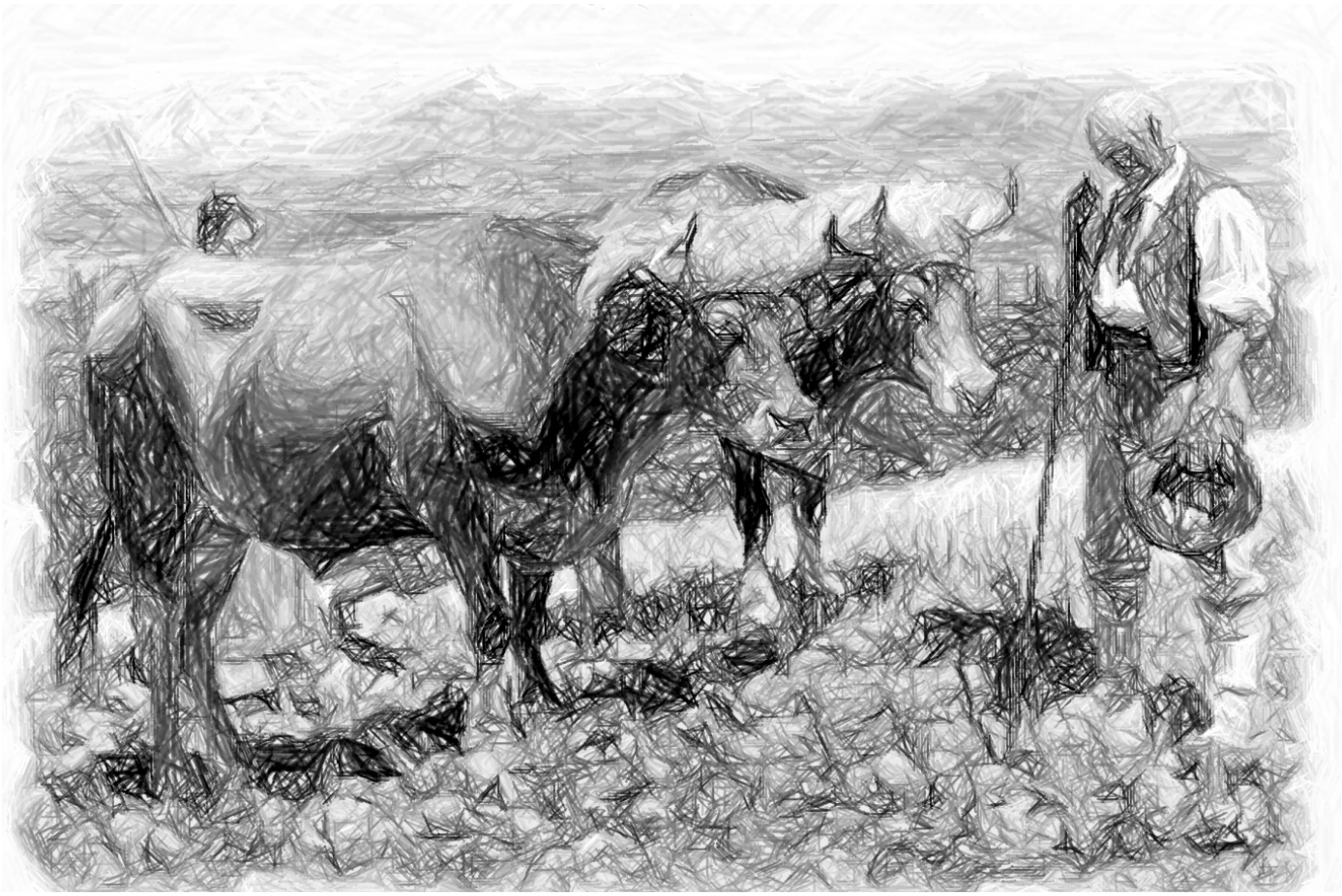
Linear regression analyses were conducted to examine the associations among the genotype of 84 bp-indel and individual FAs following a linear regression model:

$$Y_i = \beta_0 + \beta_1 \chi_{i1} + e_i$$

where Y_i is dependent variable (FAs); β_0 is overall intercept; β_1 is slope coefficient; X_i is genotype of 84 bp-indel polymorphism of *SREBP1* (SS/SL or LL); e_i is random error component.

To construct figures, regression and pearson correlation analyses between *SREBP1* gene expression and FA composition were also conducted in R (R Core Team, 2019), using the packages ggplot2 and ggally (Schloerke et al., 2020; Wickham, 2016). Pearson correlations were computed among gene expression (ΔCt) of *SREBP1* and main individual and FA groups to evaluate differences among commercial types, but also differences among the genotypes of 84 bp-indel. Partial Pearson correlations corrected by breed and sex or GE were also computed, whenever it was permitted by the sample size. Significant differences were declared at $p \leq 0.05$.

IV. RESULTS AND DISCUSSION



1. GENETIC APPROACH

Genetics has become a powerful scientific approach, which has gradually emerged and has focused in the features of nucleid acids (DNA and RNA) and the discovery of molecular markers, based on the nucleotide sequence variations within individuals. In the livestock sector, molecular genetics in conjunction with conventional animal breeding techniques have become a common strategy in animal breeding programmes. Genetic approaches are also of great importance to ensure traceability and food safety, which are relevant issues within public health.

The first part of this thesis is dedicated to the genetic characterization of native (Pirenaica and Terreña) and allochthonous (Salers and Holstein-Friesian) breeds considering their importance in local breeding programmes, management and meat production of the Basque region. The effectiveness and genetic assignments of several microsatellite marker panels for parentage and beef traceability have been studied, but also genetic diversity and genetic relationships with other European breeds (sampling I and sampling II).

1.1. GENETIC EFFICACY OF A COMMERCIAL STR MARKER SET IN CATTLE

As a preliminary study, a commercial kit for STR analysis was evaluated for the traceability and parentage testing in Terreña (n=28) and Salers (n=403) cattle populations. The commercial Bovine Genotypes™ Panel 1.2 (Finnzymes Diagnostics, Espoo, Finland) that includes 12 STRs (BM1818, BM1824, BM2113, ETH10, ETH225, ETH3, INRA023, SPS115, TGLA122, TGLA126, TGLA227, and TGLA53) was used (*Publicacion I*).

Overall, the 12 studied loci were polymorphic, but the genotypes of TGLA53 microsatellite were doubtful due to three stutters per allele and the unbalanced size of alleles. Therefore, in this preliminary study, this marker was excluded from the analysis because of the low and uncertain efficiency in genotype determination. These results related to TGLA53 marker has been also described previously (Putnova et al., 2011; Sanz et al., 2014), suggesting the need for other markers with comparable results among laboratories.

In Salers and Terreña cattle populations, HW equilibrium was respected in all markers, except for TGLA227 (**Table IV.5.**). When Salers substructure was analysed, only the

animals from a single breeder showed TGLA227 in HW disequilibrium, probably due to a certain reproductive isolation or inbreeding. Although Terreña sample was smaller than Salers sample size, Terreña breed reflected a slightly greater genetic variability (**Table IV.5.**), more than likely related to its local use and semi-wild management compared to the commercial selection in Salers.

Table IV.5. Diversity and parentage parameters of Terreña and Salers populations.

Breed	Marker	k	*Ho*	*He*	PI	PIC	PD	PE1	PE2	HW	F(Null)
TER	BM1818	6	0.643	0.523	1.40	0.490	0.7704	0.15	0.322	0.942	-0.163
	BM1824	4	0.750	0.744	2.00	0.681	0.8647	0.307	0.481	0.745	-0.010
	BM2113	10	0.929	0.869	7.00	0.837	0.9260	0.544	0.707	0.336	-0.042
	ETH10	3	0.536	0.458	1.08	0.386	0.6199	0.101	0.215	0.561	-0.104
	ETH225	5	0.643	0.766	1.40	0.708	0.8826	0.340	0.516	0.128	+0.080
	ETH3	7	0.821	0.755	2.80	0.699	0.8546	0.337	0.513	0.380	-0.051
	INRA023	10	1.00	0.867	14.0	0.834	0.9286	0.537	0.701	0.614	-0.082
	SPS115	8	0.786	0.749	2.33	0.708	0.8954	0.354	0.538	0.912	-0.034
	TGLA122	6	0.536	0.729	1.08	0.663	0.8724	0.294	0.463	0.154	+0.146
	TGLA126	5	0.714	0.697	1.75	0.630	0.8087	0.268	0.434	0.228	-0.032
	TGLA227	9	0.857	0.846	3.50	0.811	0.9337	0.498	0.669	0.936	-0.017
SAL	BM1818	7	0.340	0.324	0.76	0.313	0.5514	0.057	0.187	0.831	-0.059
	BM1824	5	0.720	0.729	1.78	0.680	0.8794	0.311	0.485	0.231	+0.006
	BM2113	9	0.730	0.710	1.85	0.682	0.8870	0.323	0.51	0.321	-0.011
	ETH10	7	0.744	0.714	1.96	0.665	0.8642	0.300	0.472	0.784	-0.013
	ETH225	5	0.772	0.770	2.19	0.731	0.9064	0.369	0.547	0.116	+0.004
	ETH3	8	0.576	0.583	1.18	0.530	0.7741	0.183	0.339	0.230	+0.011
	INRA023	8	0.695	0.731	1.64	0.698	0.8920	0.342	0.524	0.013	+0.020
	SPS115	6	0.201	0.211	0.63	0.203	0.3595	0.023	0.112	0.013	+0.062
	TGLA122	9	0.687	0.696	1.60	0.659	0.8732	0.295	0.476	0.516	+0.016
	TGLA126	5	0.588	0.590	1,21	0.503	0.7477	0.176	0.300	0.099	+0.001
	TGLA227	12	0.742	0.769	1.94	0.741	0.9097	0.396	0.576	0.000	+0.022

K: Number of alleles per locus, *Ho*: Observed homozygosity, *He*: Expected heterozygosity, PI: paternity index, PIC: polymorphic information content, PD: power of discrimination; PE1: power of exclusion for duos, PE2: power of exclusion for trios, HW: Hardy-Weinberg equilibrium, F (null): null allele frequency, TER: Terreña, SAL: Salers.

The forensic efficiency evaluation for 11 loci of this commercial multiplex kit showed higher values of combined power of discrimination in Terreña (CPD ≥ 0.9999999998) than in Salers (cPD ≥ 0.999999996). The combined paternity exclusion probabilities were $CPE_1 \geq 0.9918$ in duos and $CPE_2 \geq 0.9997$ in trios for Terreña, whereas it was $CPE_1 \geq$

0.9642 in duos and $CPE_2 \geq 0.9979$ in trios for Salers, proving the discriminatory strength of this panel (**Figure IV.16.**).

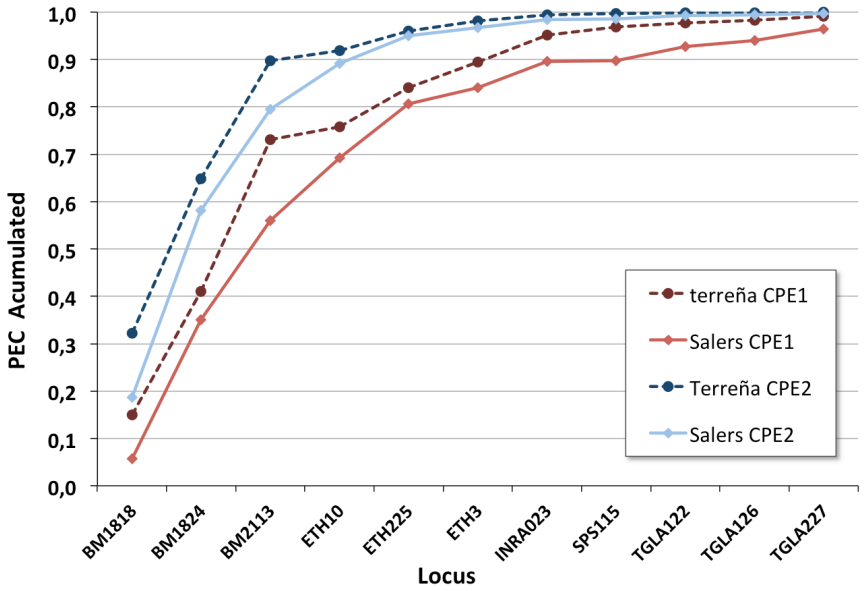


Figure IV.16. Accumulated CPE (combined power of exclusion) probabilities in the absence of genetic information from one parent (CPE1) and power of exclusion when genetic information of both parents was available (CPE2) calculated for Terreña and Salers populations.

Although the 12-STR commercial panel was recommended by ISAG (2008), one locus showed inaccurate genotyping and was excluded. The CPE_1 values revealed to be slightly not sufficient for a reliable parentage analysis. Even though these loci have been extensively used and the creation of many database justify their maintenance, the presence of the ineffective TGLA53 in the commercial kit has been questioned. However, even if some of these loci have showed low variability, it is not practical their substitution and, therefore, it would be more convenient to increase the number of loci to accomplish a suitable statistical power for parentage and traceability applications.

1.2. GENETIC CHARACTERIZATION AND FOUNDER EFFECT ANALYSIS OF A RECENTLY INTRODUCED SALERS ALLOCHTHONOUS CATTLE BREED POPULATION

Salers population has grown from few animals in Spain and a founder effect could have occurred due to the low number of initial reproducers. In the present study, the previously analysed and optimized STR commercial panel has been applied in the Spanish Salers population for: 1) genetic characterization and to evaluate whether a founder effect have occurred; 2) to understand if imported animals and good breeding management has been enough to maintain the mutation-drift equilibrium; and 3) to investigate the Salers phylogenetic relationships with other European cattle breeds (*Publication II*).

In Spanish Salers, average heterozygosity was 0.621, moderate in comparison to the values reported for other European breeds. Heterozygosity in Salers was higher than the reported by Cymbron et al. (2005) in French Salers (0.55), consistent to the value reported by Cañon et al. (2001) (0.631) and lower than the reported by Amigues et al. (2011) (0.67), although these studies were performed using other markers and with a smaller sample size.

Several publications have reported the presence of zebu and African-type microsatellite alleles in Iberian cattle breeds (Beja-Pereira et al., 2003, Ginja et al., 2010). Our study confirms the presence of African zebu-diagnostic alleles in Spanish Salers, such as BM2113-131 (0.488) and BM2113-143 (0.0645) described by MacHugh et al. (1996). These African-type alleles are consistent with an African heritage also shown in mitochondrial DNA studies. T1 haplogroup, almost fixed in African cattle, has also been found in southern European breeds (Portugal, Spain, Greece and Italy) (Bradley et al., 1996, Cymbron et al., 1999, Beja-Pereira et al., 2006) and in French Limousin (Achilli et al., 2009). This could suggest that northern African cattle may have left a genetic signature in southern Europe based on the hypothesis of Neolithic dispersal through the Mediterranean route (Zilhao, 1993) and historical migrations (Cymbron et al., 1999). According to the literature, and exploring specifically the data of Van de Goor et al. (2009) against the previous hypothesis related to migrations, zebu BM2113-131 allele was present in a small frequency in several French breeds such as Limousine (0.222),

Blonde d´Aquitaine (0.124) and Charolais (0.054), whereas the BM2113-143 allele was more frequent in French Blonde d´Aquitaine (0.167) and less frequent in Limousin (0.036) compared to our Salers sample (0.0645). These results support the hypothesis of agropastoral nomadic herding across the Mediterranean basin and reaching France and, therefore, the high frequency of zebu BM2113-131 allele could be explained by a zebu gene flow from the Mediterranean area. In fact, SNP data is in agreement with the postulated migration route by which the Neolithic culture expanded towards France (Gautier et al., 2010). Moreover, the NJ dendrogram constructed using STR data from Spanish Salers and other European breeds showed a cluster where Salers, Blonde d´Aquitaine and Limousin breeds grouped together probably due to their high frequency in African zebu allele BM2113-131 (**Figure IV.17.**).

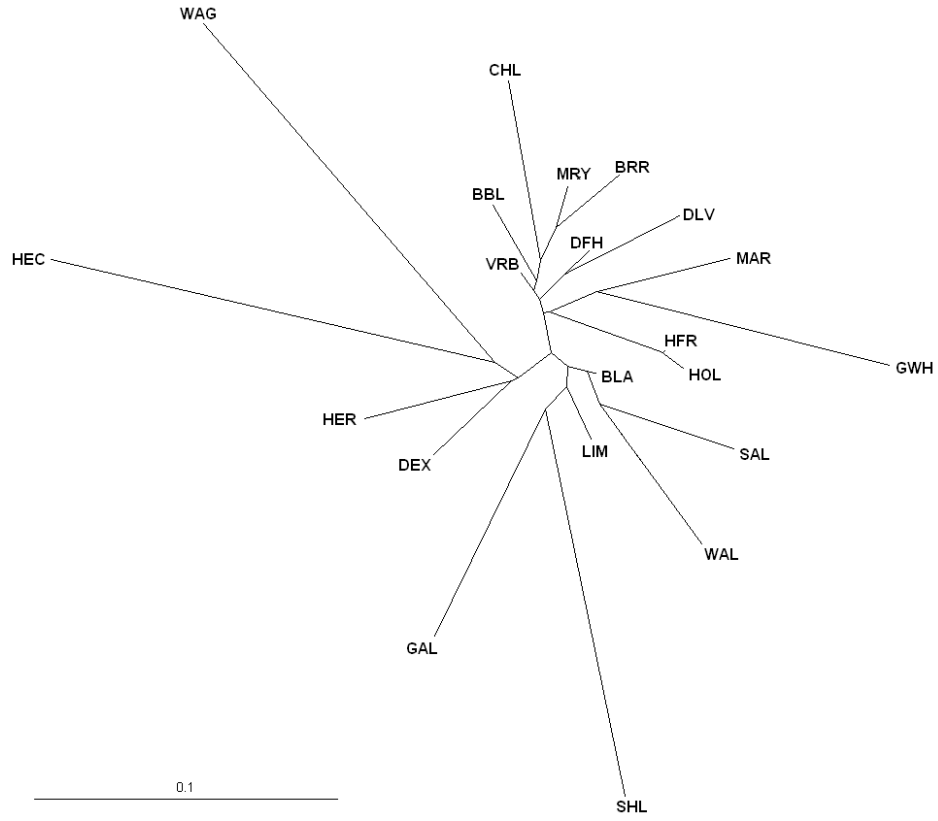


Figure IV.17. Neighbour-joining tree based on the Reynolds genetic distances between Salers (SAL) and other breeds (France: BLA, Blonde D'Aquitaine; LIM, Limousin; CHL, Charolais; Ireland: DEX Dexter; Great Britain: SHL, Scottisch Highlander; HER, Hereford; GAL, Galloway; Belgium: BBL, Belgian Blue; Austria: WAL, Waldviertler Blondvieh; Italy: MAR, Marchigiana; Netherlands: HFR, Holstein Friesian; HOL, Holstein; DFH, Dutch Friesian; GWH, Groningen Whiteheaded; DLV, Dutch Belted; MRY, Maas Rijn Ijssel; VRB, Verbeterd Roodbont; BRR, Brandrood; Outlier Japanese breed: WAG, Wagyu).

A potential genetic founder effect in Salers population cannot be discarded since the number of Salers individuals initially coming from France was very low. Sign test and Wilcoxon sign rank test were performed under Stepwise Mutation Model (SMM) and two-phased model of mutation (TPM) (**Table IV.6.**). Sign test showed heterozygosity excess in 2 loci under SMM (P = 0.007) and TPM (P = 0.008) and revealed significant deviation under SMM and TPM models (uncorrected). Sign test showed low statistical power (Piry et al., 1999), but after Bonferroni correction ($p < 0.0045$), none of models showed significant deviation. Using the Wilcoxon rank test, a non-parametric test with relatively high power, the probability values were not significant under either models, rejecting a founder effect in both cases.

Table IV.6. Different tests for founder effect analysis in Salers cattle.

Test		TPM	SMM
Sign test	Number of loci with H_e excess	2	2
	Number of loci with H_e deficiency	9	9
	P value	0.008	0.007
Wilcoxon sign rank test	P value (one-tail test for H_e excess)	0.517	0.994

H_e = Heterozygosity

The Mode-shift indicator test was also utilized as an additional method to detect a potential founder effect and a normal L-shaped graphical representation where most abundant alleles showed low frequencies (0.01-0.1) (*Publication II*), suggesting that studied population is under mutation-drift equilibrium. Therefore, even Salers breed has been recently introduced in Spain from France, constant incorporation of animals for breeding could have increased the effective population avoiding negative effects from reduced founder population and inbreeding. Overall, the high genetic diversity in Spanish Salers highlights an adequate potential to respond to future breeding and selection programs.

To test the genetic substructure, the STR data of our Salers population and other genetic data of other European cattle breeds (France: Blonde D'Aquitaine, Limousin, Charolais; Ireland: Dexter; Great Britain: Scottish Highlander, Hereford, Galloway; Belgium: Belgian Blue; Austria: Waldviertler Blondvieh; Italy: Marchigiana; Netherlands: Holstein

Friesian, Holstein, Dutch Friesian, Groningen Whiteheaded, Dutch Belted, Maas Rijn Ijssel, Verbeterd Roodbont, Brandrood; Outlier Japanese breed: Wagyu) described in Van de Goor et al. (2009) (*Publication II*). Differences in Reynolds genetic distances for each pair of breeds were mostly significant ($p < 0.001$), meaning that the breeds can be considered genetically independent. Clustering analysis using a Bayesian MCMC approach showed that Salers breed does not show a partitioning of genetic variability according to geographical origin (**Figure IV.18.**). Structure's posterior probabilities of K, as a log-likelihood given K clusters and the corresponding ΔK statistic (Evanno et al., 2005) showed maximal ΔK occurred at K=2 and 95.9% of animals were attributed to one of the two clusters using a cutoff value of 80% ancestry for assignment. Structure from Amigues et al. (2011) differentiated Salers, Blonde d´Aquitaine and Limousin at K=4. In contrast, our results showed that Blonde d´Aquitaine, Limousine and Charolais seems to be more related as observed in the bayesian clustering and the major clusters of the NJ tree based in genetic distance analysis (**Figure IV.18.**).

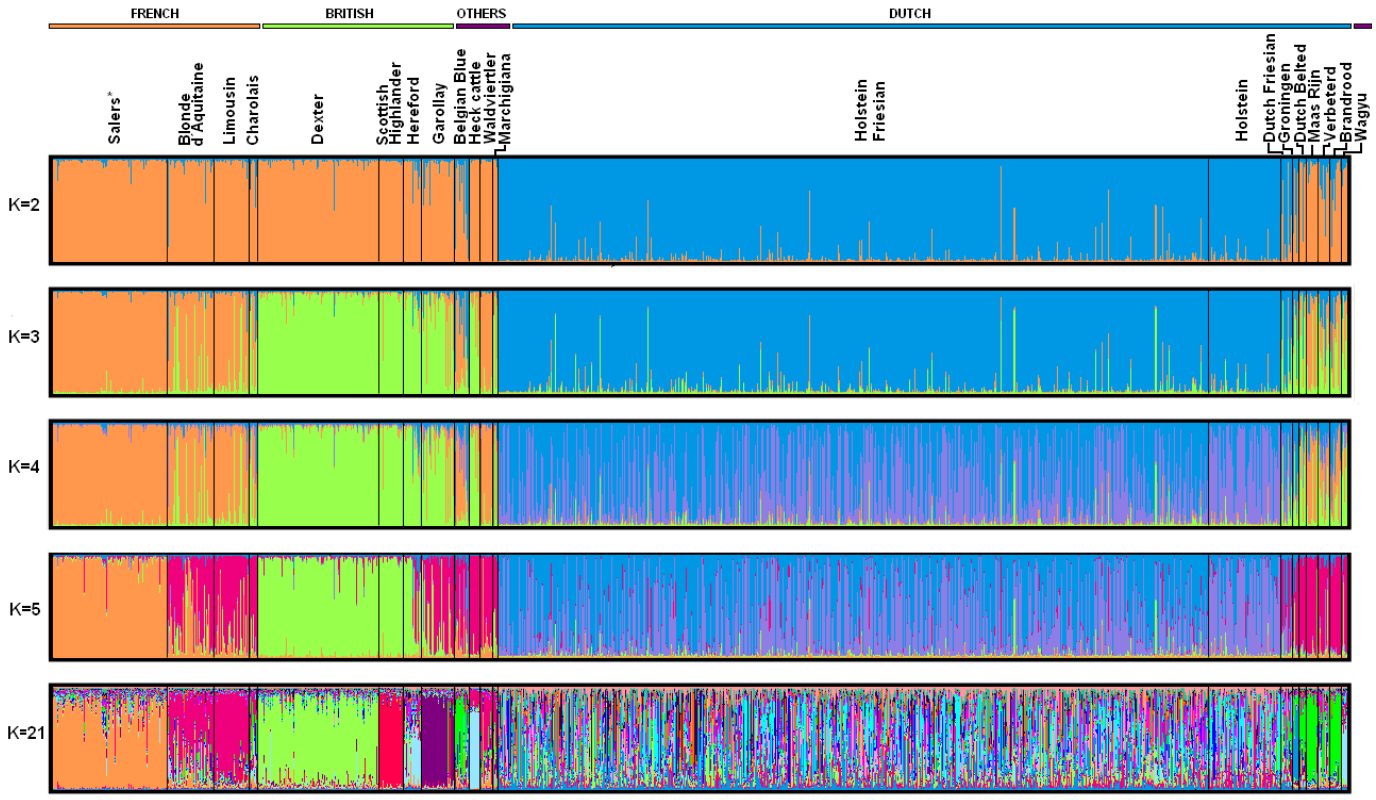


Figure IV.18. Population partitioning suggested by Bayesian Markov Chain Monte Carlo approach. Each individual animal is represented by a single vertical line divided into K colours, where K is the number of clusters assumed, and coloured segments represent the individual's estimated proportion of membership to a particular cluster. Black lines separate individual populations which names are indicated above the diagram.

Finally, a correspondence analysis plot was performed including all animals, in which a total of 9.45% of the variance accounted for the first three dimensions (**Figure IV.19.**). Axis 1 separates Holstein Friesian and Dutch breeds (positive: green and black) from France and British islands breeds (negative). Axis 2 separates French breeds (positive: yellow and blue) and British breeds (negative: purple). French breeds seemed to be closer and separated from British breeds. British breeds showed high differentiation probably caused by their island isolation. On the other hand, other breeds were dispersed on the centre of the plot but close to a big cluster of Holstein Friesian. In general, populations were mostly separated according to their geographical origin, although some exceptions were observed.

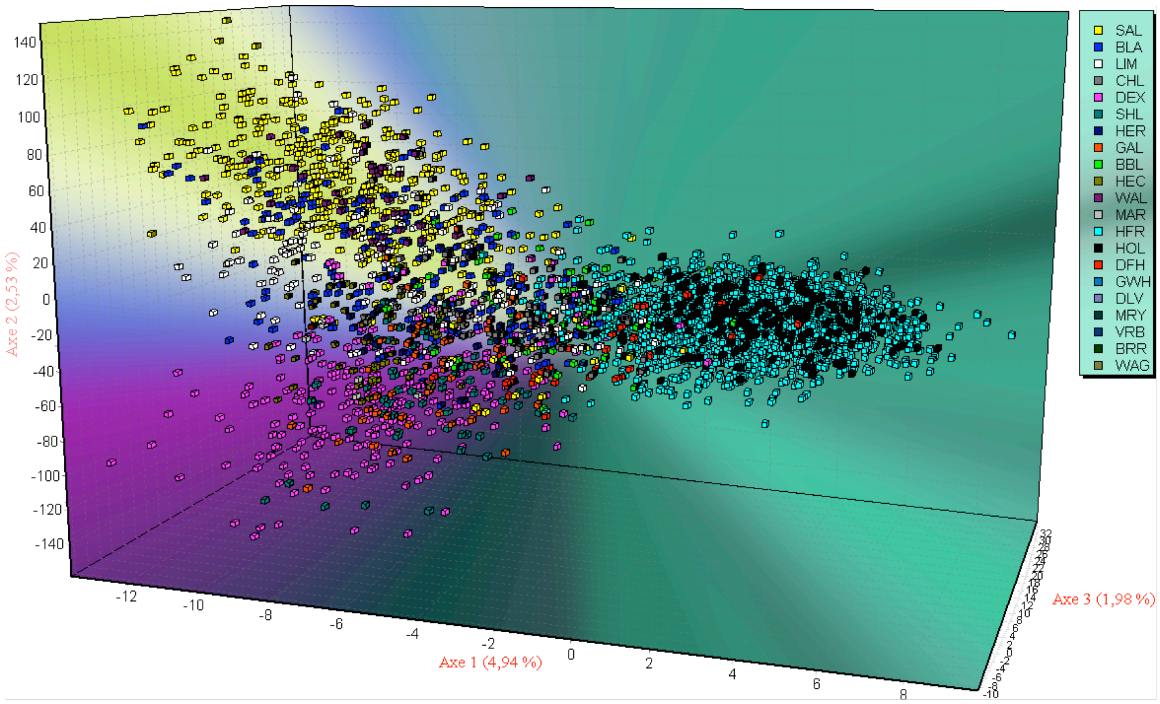


Figure IV.19. Plot of multivariate correspondence analysis showing the distribution of individual animals of all populations.

1.3. GENETIC CHARACTERIZATION OF PIRENAICA AUTOCHTHONOUS CATTLE BREED FOR PARENTAGE AND TRACEABILITY PURPOSES

In the previous studies, a commercial STR panel demonstrated to be applicable for parentage, identification and genetic diversity studies (*Publication I & II*). However, according to those results, an increase in the amount of STR markers can provide a trustworthy traceability in the meat industry. Moreover, the genetic characterization of native Pirenaica is of great importance as local AnGR of the Basque Country. Taking all these into account, an appropriate STR panel for Pirenaica, and also applicable to other native and allochthonous breeds, is necessary for appropriate livestock management and further meat traceability in the commercial supply chain. In this regard, in the present section, the genetic effectiveness of the ISAG/FAO 30-STR panel was assessed for parentage, individual identification and selection of the most discriminative markers against other breeds typically reared in the Basque Country (Pirenaica, Terreña, Blonde d´Aquitaine, Limousin, Salers and Holstein-Friesian). Additionally, the genetic diversity and phylogeny of Pirenaica cattle together with other breeds was studied.

Overall, the average heterozygosity value in Pirenaica (n=114; 0.680) was slightly higher (**Table IV.7.**) than values reported in other native breeds from Spain, Portugal and France ranging from 0.50 to 0.71 (Maudet et al., 2002). Compared to other studies in Pirenaica, the value was similar to the one reported by Rendo et al. (2004) (n=56; 0.688) and higher than values reported by Martin-Burriel et al. (1999) (n=50; 0.617) and Cañon et al. (2001) (n=50; 0.628).

Table IV.7. Statistical parameters for genetic characteristics of Pirenaica breed using a panel of 30 STRs

Marker	k	*Ho*	*He*	PIC	MP	PE-1P	PE-2P	PE-SI	HW
BM1824	4	0.711	0.745	0.694	0.114	0.319	0.668	0.592	NS
BM2113	8	0.816	0.824	0.797	0.062	0.469	0.821	0.646	NS
ETH03	8	0.588	0.621	0.582	0.181	0.224	0.597	0.513	NS
ETH010	6	0.746	0.710	0.658	0.160	0.295	0.649	0.570	NS
ETH225	6	0.684	0.639	0.604	0.161	0.241	0.623	0.527	NS
INRA023	7	0.737	0.769	0.729	0.088	0.373	0.736	0.610	NS
SPS115	6	0.596	0.581	0.522	0.235	0.182	0.503	0.481	NS
TGLA53	11	0.754	0.820	0.791	0.063	0.462	0.815	0.643	NS
TGLA122	11	0.746	0.768	0.734	0.084	0.387	0.758	0.611	NS
TGLA126	5	0.658	0.608	0.556	0.201	0.203	0.545	0.502	NS
TGLA227	11	0.860	0.865	0.845	0.039	0.559	0.882	0.672	NS
BM1818	7	0.728	0.783	0.750	0.080	0.401	0.770	0.620	NS
CSSM66	12	0.732	0.777	0.755	0.078	0.422	0.808	0.619	NS
CSRM60	7	0.652	0.632	0.602	0.163	0.240	0.633	0.523	NS
ILSTS006	7	0.719	0.806	0.774	0.070	0.432	0.789	0.634	NS
HAUT27	7	0.693	0.745	0.702	0.111	0.342	0.706	0.594	NS
MM12	11	0.842	0.804	0.777	0.086	0.445	0.810	0.634	NS
HEL09	7	0.754	0.798	0.764	0.075	0.417	0.776	0.629	NS
INRA032	8	0.693	0.747	0.710	0.099	0.351	0.725	0.597	NS
ETH152	6	0.788	0.746	0.701	0.124	0.336	0.696	0.594	NS
HAUT024	8	0.772	0.737	0.691	0.123	0.327	0.687	0.589	NS
INRA037	9	0.268	0.663	0.601	0.231	0.250	0.588	0.536	***
INRA005	4	0.604	0.642	0.564	0.198	0.206	0.500	0.518	NS
ETH185	8	0.536	0.596	0.554	0.222	0.202	0.563	0.496	NS
HEL05	8	0.561	0.567	0.535	0.217	0.185	0.555	0.477	NS
INRA063	4	0.563	0.576	0.506	0.243	0.172	0.469	0.474	NS
HEL01	5	0.554	0.558	0.505	0.254	0.165	0.486	0.466	NS
HEL13	5	0.482	0.469	0.421	0.352	0.113	0.400	0.401	NS
INRA035	3	0.298	0.427	0.384	0.397	0.091	0.357	0.370	NS
ILST005	2	0.351	0.372	0.302	0.463	0.069	0.237	0.319	NS

k: number of alleles per locus; Ho: observed homozygosity; He: expected heterozygosity; PIC: polymorphic information content; MP: matching probability; PE-1P: power of exclusion for 1 known parent; PE-2P: power of exclusion for 2 known parents; PE-SI: power of exclusion for sibling; HW: Hardy–Weinberg equilibrium; NS: non-significant; ***, $p < 0.001$.

Based on heterozygosity and the mean PIC value of 0.637, Pirenaica showed a slightly lower genetic diversity than other Basque breeds such as Terreña, Monchina and Betizu (Rendo et al., 2004) and than the diversity showed in our previous Terreña study (*He*: 0.846; *Publication I*). This agrees well with the recovery and breeding programmes applied to Pirenaica, whereas Terreña has been kept in a semi wild natural environment.

The origin of Iberian breeds happened with the arrival of cattle from the *Bos taurus* (Taurine) lineage. In Pirenaica (taurine) breed, the zebu diagnostic 131-bp allele of the BM2113 marker showed a frequency of 0.149. Other zebu diagnostic alleles were also present at very low frequencies such as ETH152-193 (0.075) and BM2113-123 (0.009). The results based in BM2113-131 allele, observed in Pirenaica, agree with zebu and African-type STR alleles previously reported in Iberian cattle (Beja-Pereira et al., 2003, Ginja et al., 2010). Other studies also reported an African mitochondrial T1 haplogroup in Pirenaica cattle (Lopez-Oceja et al., 2016). As previously discussed, these zebu markers may suggest a northern African genetic signature based on the hypothesis of Neolithic dispersal through the Mediterranean basin (Zilhao et al., 1993, Cymbron et al., 1999), which reached to the Basque region during the historical human and livestock migrations. In this regard, zebu alleles observed in Pirenaica and Terreña, but also in Salers (*Publication II*), indicate an African genetic signature not only in the Mediterranean basin but also in farther regions and other southern European cattle breeds.

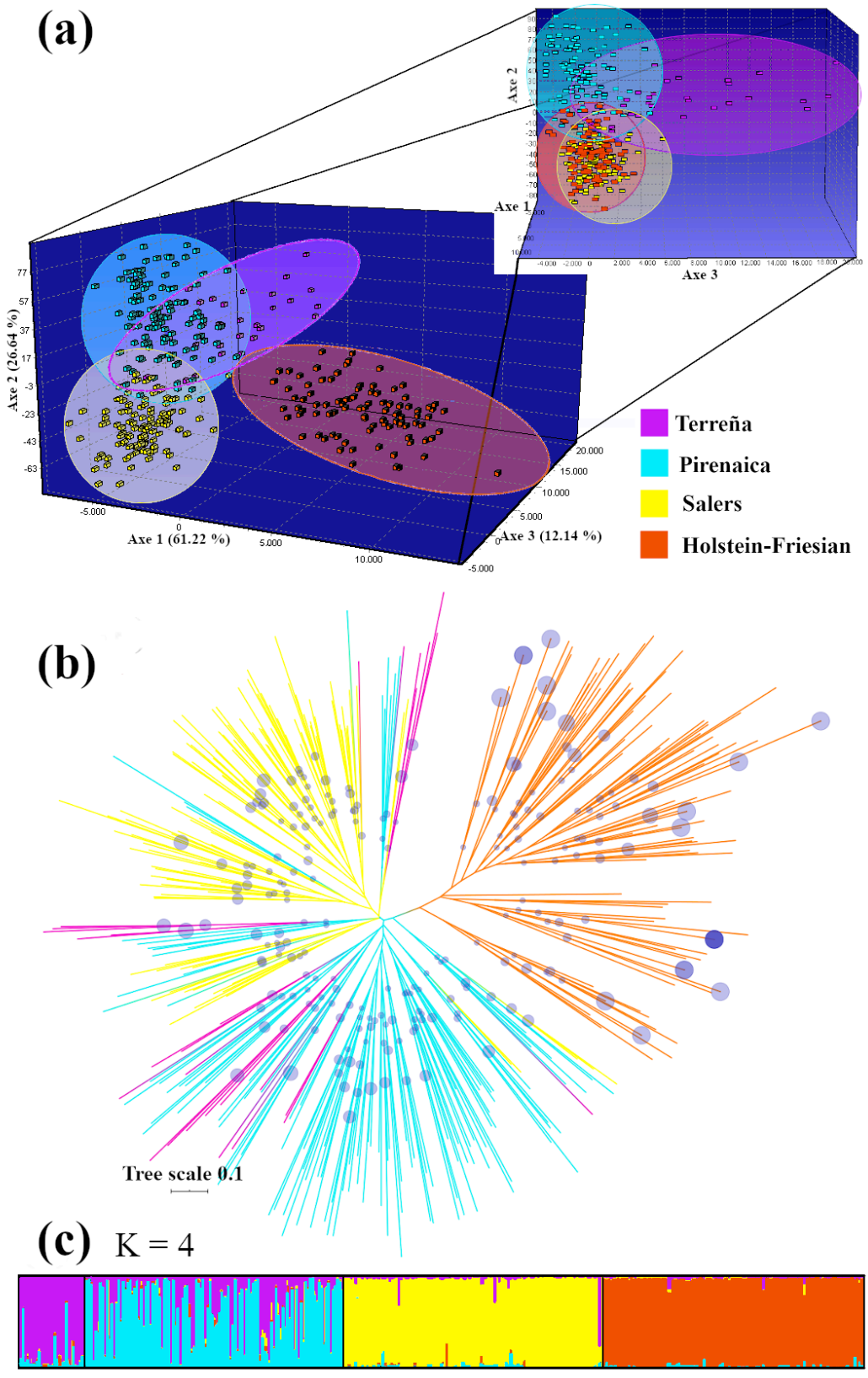


Figure IV.20. Genetic variations and phylogenetic differentiation analyses among cattle breeds. (a) 3D-FCA, (b) NJ radiation tree (Scale measured in R_{ST} distance values), size of circles represents bootstraps percentage and (c) mean probabilities of individual cluster memberships using structure (K = 4).

The relationship between Pirenaica (turquoise) and other breeds managed in the Basque Country such as Basque native Terreña (purple), Salers (yellow) and Holstein-Friesian (orange) showed population differentiation; the breeds were significantly different from each other ($p < 0.001$; **Figure IV.20.**). Pirenaica and Terreña breeds, both native from the Basque region, showed certain admixture. In contrast, allochthonous Salers and Holstein-Friesian were well differentiated from Pirenaica and Terreña, corresponding to their distant geographical origin in other European regions (**Figure IV.20**). The overall genetic differentiation among breeds (F_{ST}) was high (0.1195; **Table IV.8.**), while it affected the performance of assignment tests. It is known that genetically divergent breeds ($F_{ST} > 0.1$) are more likely to be correctly assigned than closely related ones ($F_{ST} < 0.05$) (Maudet et al., 2002).

Table IV.8. Pairwise F_{ST} (below diagonal) and R_{ST} (above diagonal) between breeds of cattle

	Terreña	**Pirenaica**	**Salers**	**Holstein-Friesian**
Terreña	-	0.04277	0.14579	0.14030
Pirenaica	0.04102	-	0.11512	0.14115
Salers	0.12724	0.10324	-	0.24822
Holstein-Friesian	0.12304	0.12369	0.19886	-

All F_{ST} and R_{ST} values are significant ($p < 0.001$).

The effectiveness of a 30-STR panel, recommended by ISAG-FAO (2011), has been investigated for parentage and individual identification purposes and the results were compared to other commercial STR panels. In Pirenaica, the CMP value was 1.89×10^{-25} when 30 markers were used, while ISAG core panel of 12 STRs available in Genotype Panel 1.2 (Thermo Scientific) showed a CMP value of 2.3×10^{-11}.

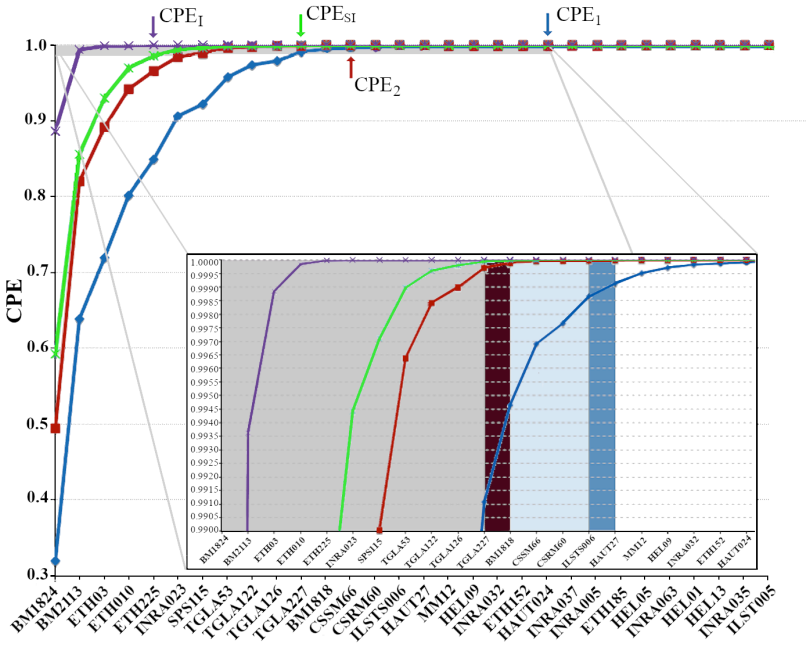


Figure IV.21. Comparison of combined power of exclusions (CPE) between the panels of STR markers. STRs considering one known parent, two known parents, sibling and identical are shown in blue (CPE_1), red (CPE_2), green (CPE_{SI}), and purple ($CPE_I = 1 - CMP$) cases, respectively. Little arrows (top) show the minimal number of markers for 99.99% of CPE. Boxes (below) show the markers included in recommended and commercial STR panels (grey, Stockmarks; garnet, ISAG core; blue, Bovine genotype 1.2 and 2.2).

In parentage testing of Pirenaica, the Combined Power of Exclusion in the absence of genetic information from one parent (CPE_1) and when genetic information of both parents was available (CPE_2) was computed considering all 30 loci. Also, reference studies using STR panels with less loci were taken into account. Using 11 STRs, Pirenaica showed similar CPE_1 (0.9911; **Figure IV.21.**) to Terreña (0.9918; *Publication I*), while CPE_2 was 0.9997 in both breeds. When using the minimum 12-STR core panel recognized by ISAG, Pirenaica had a CPE_1 and CPE_2 value of 0.9946 and 0.9998, respectively. In other European breeds, 12-STR panel showed CPE_1 values that ranged from 0.9135 to 0.9777, and CPE_2 values that ranged from 0.9935 to 0.9999 (Van de Goor et al., 2009). However, a CPE value over 0.9999 is necessary for paternity analysis, and, therefore, the 12-STR panel might be insufficient depending on breed and parentage. This evidenced the need to increase the number of STR loci in Pirenaica, in order to have enough exclusion power to resolve satisfactorily parentage cases.

The recommended 30-STR panel, used in this study, showed a CPE_1 (considering one known parent) and CPE_2 (two known parents) values of 0.99998 and 0.99999997, respectively. Our Pirenaica results showed that a 21-STR panel might be necessary to

obtain a CPE_1 value of 0.9999, while a 13-STR panel is enough for a discriminative CPE_2 value of 0.9999 (**Figure IV.21.**). Overall, for most forensic cases, except CPE_{SI}, the 12-STR core panel seems insufficient in Pirenaica and an increase in the amount of genotyped STRs should be considered. In this regard, a 21-STR panel looks more appropriate to resolve some parentage cases in Pirenaica.

The assignments of GENECLASS showed that over 99% of individuals were allocated within their populations using both frequency (99.67%) and Bayesian (99.70%) methods, whereas the Bayesian assignment of STRUCTURE showed that the overall proportion of animals correctly assigned to a breed was 98.8%,considering the 30-STRs panel. Using Terreña and Pirenaica 11-STR data, Pirenaica assignment decreased to 66.1%. Therefore, a STRUCTURE assignment test suggested that the number of STRs should be increased when other close native breeds were included in the data set. GENECLASS or STRUCTURE methods encounter several limitations. The frequency-based method lacks the *p* value for measuring the confidence with which an individual belongs to a given population. Whereas, previous Bayesian methods can bias assignments or provide inaccurate results if sample sizes are unbalanced among populations (Wang, 2017). In order to overcome the problem of unbalanced population sizes, a machine-learning approach was applied to study the mean and variance of assignments (**Figure IV.22.**). First, with an 11-STR panel, low means of assignment were observed in Pirenaica (62.9%) and Terreña (58.2%), with high variance in the bar-plots due to the non-accuracy of the assignment tests (**Figure IV.22.a**). Thus, an 11-STR panel might not be enough for an accurate assessment of an individual to its population of origin, when geographically and phylogenetically related native Pirenaica and Terreña breeds are studied. With the 16-STR panel, high mean assignment values were observed in Pirenaica (98.9%) in comparison to Blonde d'Aquitaine (45.4%) and Limousin (54.1%) (**Figure IV.22.b**). Finally, Pirenaica and Salers showed over 90% mean assignments for the 16, 21 and 30-STR panels, while percentages were lower for the 11-STR panel with 70.4% and 83.5%, respectively (Figure 4.8.c). In contrast, for Holstein-Friesians a 21-STR panel was required to reach an assignment of 90% probably related to a reduced genetic diversity related to selection for milk production. In general, 11 or 12-STR commercial panels seem insufficient for reliable assignment tests even selecting highest F_{ST} markers. Pirenaica showed that a panel of over 21-STRs is necessary for trustworthy assignments (≥95%; *Publication III*). In essence, a reliable molecular traceability to ensure a correct assessment of an unknown beef product to its origin PGI label will depend on a well-designed STR panel containing the minimum amount of STRs with the highest F_{ST} values.

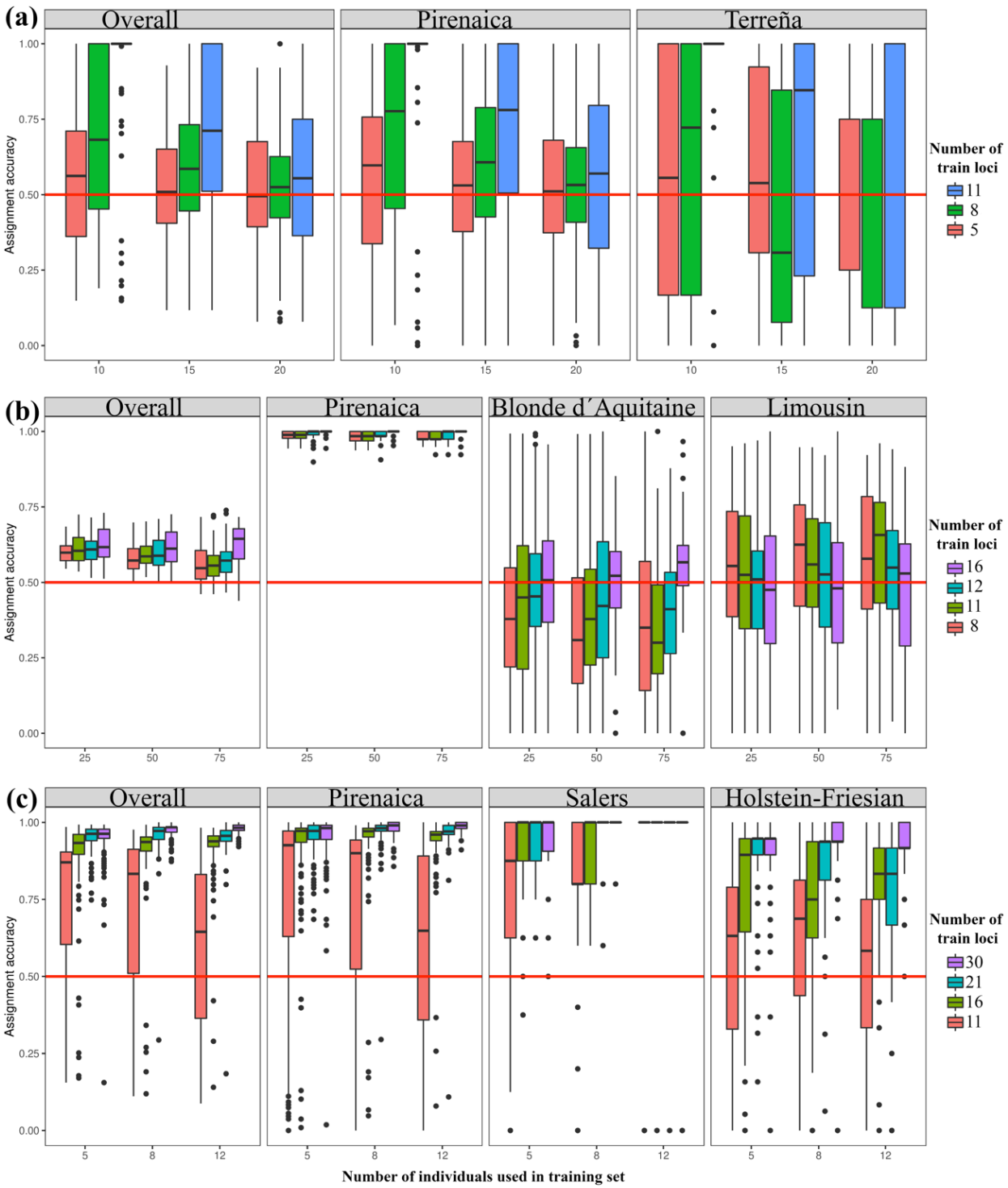


Figure IV.22. Assignment accuracies (%), according to Monte-Carlo cross-validation and depicted as bar-plots. (**a**) Pirenaica and Terreña breeds' assignments for balanced populations (10, 15 and 20 individuals) crossed by three levels of train STRs with the highest F_{ST} (red: 5-STR; green: 8-STR) and all loci (blue: 11-STR), (**b**) Pirenaica, Blonde d´Aquitaine and Limousin breeds' assignments for balanced populations (25, 50 and 75 individuals) crossed by four levels of train STRs with the highest F_{ST} (red: 8-STR; green: 11-STR; turquoise: 12-STR) and all loci (fuchsia: 16-STR). (**c**) Pirenaica, Salers and Holstein-Friesian breeds' assignments for balanced populations (5, 8 and 12 individuals) crossed by four levels of train STRs with the highest F_{ST} (red: 11-STR; green: 16-STR; turquoise: 21-STR) and all loci (fuchsia: 30-STR).

2. MEAT QUALITY APPROACH: FATTY ACID COMPOSITION AND ITS RELATIONSHIP WITH GENETICS

This second part of the Ph.D. Thesis is focused on the FA characterization of cattle and its relationship with lipogenic genes. This is the first time where genetics of cattle breeds reared in the Basque region have been related to meat quality parameters; concretely FA profile. Up to date, the mechanisms by which lipogenic genes affect the FA composition and the related biochemical pathways are not fully understood. Therefore, this part is dedicated to investigate the expression level and variability of several lipogenic genes and their associations with the FA composition of subcutaneous fat from major commercial cattle breeds reared in the Basque Country (sampling II).

2.1. FATTY ACID COMPOSITION AND GENE EXPRESSION IN CATTLE

Fat deposition and the FA composition of fat depots are controlled by a complex regulatory system including lipogenesis and lipolysis pathways. Adipose tissue is the main site for excess energy storage in the form of triacylglycerols, with Δ9-desaturase products being some of the predominant FAs (Mauvoisin & Mounier, 2011). It is known that the regulation of *SCD* and *SREBP1* genes strongly affects the FA composition of backfat, and therefore, this fat tissue was investigated in three genetically diverse bovine breeds reared and commercialized in the Basque Country and belonging to four commercial types: Pirenaica bulls and heifers, Salers bulls and Holstein-Friesian cows (Aurtenetxe et al., 2017).

First of all, the FA composition of collected backfat tissue was determined and several significant differences, as observed in **Table IV.9.**, were found among commercial types.

Table IV.9. Comparisons of fatty acid composition (mg/g of subcutaneous fat) and carcass parameters among commercial types.

	Commercial type				
	Salers bulls (n=13)	Pirenaica bulls (n=37)	Pirenaica heifers (n=29)	Holstein-Friesian cows (n=21)	*p*-value
Conformation	8.45 ± 0.37^{b}	10.9 ± 0.3^{a}	11.0 ± 0.3^{a}	2.02 ± 0.64^{c}	<0.001
Fatness	5.79 ± 0.46^{b}	4.52 ± 0.33^{c}	7.47 ± 0.33^{a}	1.96 ± 0.81^{d}	<0.001
14:0 ^{s1}	31.6 ± 2.4^{ab}	30.5 ± 1.7^{b}	35.6 ± 1.8^{a}	16.0 ± 4.2^{c}	<0.001
15:0 ^{s2}	4.71 ± 0.33	4.07 ± 0.23	3.99 ± 0.24	3.13 ± 0.57	0.111
16:0 ^{s3}	230 ± 11^{ab}	217 ± 8^{b}	246 ± 8^{a}	170 ± 20^{bc}	0.002
17:0 ^{s4}	9.01 ± 0.71	7.48 ± 0.50	8.08 ± 0.51	5.54 ± 1.22	0.057
18:0 ^{s5}	131 ± 11	119 ± 8	98.0 ± 8.4	129 ± 20	0.059
19:0 ^{s6}	0.565 ± 0.074^{a}	0.595 ± 0.052^{a}	0.38 ± 0.05^{b}	0.708 ± 0.129^{a}	0.005
20:0 ^{s7}	0.907 ± 0.119^{ab}	0.816 ± 0.084^{b}	0.468 ± 0.085^{c}	1.34 ± 0.21^{a}	<0.001
9*c*-14:1 ^{p1}	8.05 ± 1.27^{b}	7.80 ± 0.90^{b}	12.4 ± 0.9^{a}	1.46 ± 2.21^{c}	<0.001
9*c*-15:1 ^{p2}	0.208 ± 0.028	0.183 ± 0.020	0.206 ± 0.020	0.15 ± 0.05	0.572
9*c*-16:1 ^{p3}	36.7 ± 3.7^{b}	33.5 ± 2.6^{b}	45.3 ± 2.7^{a}	9.74 ± 6.51^{c}	<0.001
9*c*-17:1 ^{p4}	6.73 ± 0.44^{a}	5.24 ± 0.31^{b}	6.96 ± 0.32^{a}	2.15 ± 0.77^{c}	<0.001
9*c*-18:1 ^{p5}	308 ± 14^{a}	261 ± 10^{b}	333 ± 10^{a}	196 ± 24^{c}	<0.001
9*c*-19:1 ^{p6}	0.987 ± 0.055^{a}	0.789 ± 0.039^{b}	0.801 ± 0.040^{b}	0.795 ± 0.096^{ab}	0.004
9*c*-20:1 ^{p7}	0.726 ± 0.075	0.614 ± 0.053	0.728 ± 0.054	0.777 ± 0.130	0.231
6-8*t*-18:1 ^{s8}	3.14 ± 0.38^{bc}	3.66 ± 0.27^{ab}	4.12 ± 0.28^{a}	1.83 ± 0.67^{c}	0.010
11*t*-18:1 ^{s9}	10.3 ± 2.0	12.1 ± 1.4	8.20 ± 1.47	6.15 ± 3.55	0.162
12*t*-18:1 ^{s10}	2.26 ± 0.28	2.56 ± 0.20	2.71 ± 0.21	1.59 ± 0.50	0.174
13*t*/14*t*-18:1 ^{s11}	4.37 ± 0.51^{b}	5.23 ± 0.36^{ab}	5.80 ± 0.37^{a}	3.67 ± 0.89^{ab}	0.029
15*c*-18:1 ^{s12}	1.08 ± 0.16^{c}	1.38 ± 0.11^{b}	2.07 ± 0.11^{a}	0.596 ± 0.271^{c}	<0.001
7*t*,9*c*-18:2 ^{p8}	0.813 ± 0.116^{bc}	0.845 ± 0.082^{b}	1.25 ± 0.08^{a}	0.295 ± 0.201^{c}	<0.001
9*c*,11*t*-18:2 ^{p9}	3.11 ± 0.53	3.26 ± 0.376	3.53 ± 0.38	1.80 ± 0.92	0.453
9*c*,12*t*-18:2 ^{p10}	0.520 ± 0.067^{b}	0.608 ± 0.048^{b}	0.854 ± 0.048^{a}	0.312 ± 0.118^{b}	<0.001
9*c*,13*t*-18:2 ^{p11}	0.963 ± 0.131^{b}	1.07 ± 0.09^{b}	1.61 ± 0.09^{a}	0.567 ± 0.229^{b}	<0.001
9*c*,15*c*-18:2 ^{p12}	0.461 ± 0.044^{b}	0.341 ± 0.031^{c}	0.587 ± 0.032^{a}	0.266 ± 0.077^{bc}	<0.001
10*t*,12*c*-18:2 ^{i}	0.221 ± 0.041^{b}	0.195 ± 0.029^{b}	0.324 ± 0.030^{a}	0.099 ± 0.072^{b}	0.001
SFA	410 ± 21	382 ± 15	395 ± 15	328 ± 37	0.285
MUFA	427 ± 18^{b}	379 ± 13^{c}	489 ± 13^{a}	245 ± 32^{d}	<0.001
***cis*-MUFA**	385 ± 18^{b}	331 ± 13^{c}	430 ± 13^{a}	221 ± 32^{d}	<0.001
***trans*-MUFA**	42.2 ± 5.2^{bc}	48.3 ± 3.6^{b}	58.9 ± 3.7^{a}	23.5 ± 9.0^{c}	0.001
CLA	4.92 ± 0.56^{bc}	5.22 ± 0.40^{b}	6.42 ± 0.40^{a}	2.68 ± 0.98^{c}	0.002
PUFA	29.9 ± 2.1^{a}	24.3 ± 1.5^{bc}	24.4 ± 1.5^{b}	14.9 ± 3.7^{c}	0.009
n-6	27.7 ± 2.0^{a}	22.2 ± 1.4^{b}	22.1 ± 1.4^{b}	12.8 ± 3.4^{c}	0.005
n-3	2.09 ± 0.19	2.05 ± 0.14	2.15 ± 0.14	2.04 ± 0.33	0.942

Least square means ± standard deviations. SFA: summaroty of saturated fatty acids; MUFA: summatory of all, *cis* (*c*) and *trans* (*t*), monounsaturated fatty acids; CLA; summatory of

conjugated linoleic acid isomers; PUFA: summatory of polyunsaturated fatty acids.[s] substrate; [p] product; [1-12]Same superscript numbers indicate the substrate-product pairs.
[i] inhibitor of SCD enzyme (Baumgard et al., 2002) .
[a,b,c] Values within a row with different superscripts differ significantly at $P < 0.05$.

In terms of the relative mRNA expression levels of the lipogenic genes *SREBP1*, *SCD1*, and SCD5, these were similar in Pirenaica bulls and heifers that may be partially explained by a similar feeding regimen (**Figure IV.23.**). Overall, *SCD1* expression was higher than *SREBP1* expression and *SCD5* expression in all studied breeds ($p < 0.001$).

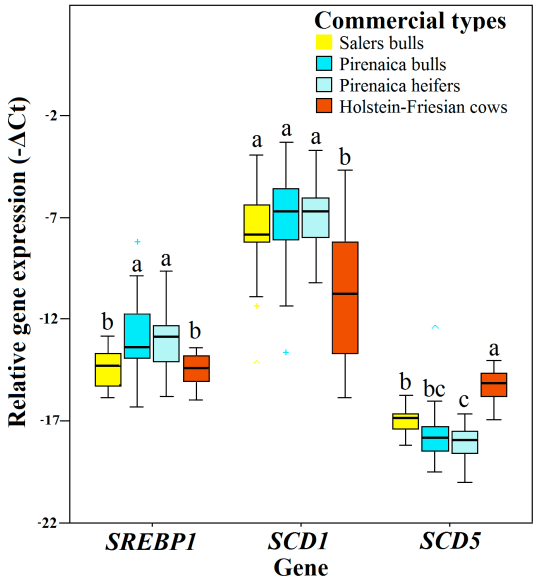


Figure IV.23. Box-plot showing the relative expression levels of *SCD1*, *SCD5* and *SREBP1* in subcutaneous fat samples from the cattle commercial types Salers, Pirenaica bulls, Pirenaica heifers and Holstein-Friesian heifers. The middle line in the box represent the mean, upper and lower areas of the center box indicate the 75th and 25th percentiles respectively, and vertical bars indicate standard errors. Differences among commercial types are indicated by different letters ($p < 0.05$).

Lengi and Corl (2007) also reported over 40-fold greater expression of *SCD1* compared to *SCD5* in adipose tissue of bulls. In general, variation among breeds was observed in the expression of both *SCD* isoforms, especially between beef and dairy cattle breeds (Salers and Pirenaica *versus* Holstein-Friesian), suggesting that even if FA differences are generally small, there may still be differences in the underlying lipogenic gene expression or enzyme profile (De Smet et al., 2004). Higher differences in expression levels of *SCD5* also suggested that *SCD5* expression is more breed dependant or more sensitive than

SCD1 expression to other environmental factors (*i.e.*, feeding) that differ among studied commercial types.

All commercial types showed strong correlations between *SCD1* and *SREBP1* (**Figure 4.24.a**). In Pirenaica heifers, *SCD1* and *SREBP1* correlation was higher than in Pirenaica bulls, suggesting that the FA composition is affected by the lipogenic gene regulation in a gender-dependant manner. Likewise, in other crossbred cattle, heifers exhibited higher *SCD1* mRNA levels and higher MUFA content than bulls in subcutaneous fat (Barton et al. 2011) and, therefore, a possible effect of sex hormones on enzymatic systems affecting lipid metabolism has been suggested (Zhang et al., 2010). *SREBP*1 and *SCD1* gene expressions were also higher in females due to the growth hormone according to Améen et al. (2004). On the other hand, age and diet also seem to influence adipocyte development in Pirenaica bulls (Soret et al., 2016). Hence, the activation of *SCD1* due to a potentially higher concentrate consumption (Smith et al., 2009) agrees with the greater total MUFA content in Salers and Pirenaica, while the higher MUFA content in Pirenaica heifers than bulls seems to be sex-dependant (**Table IV.9.**).

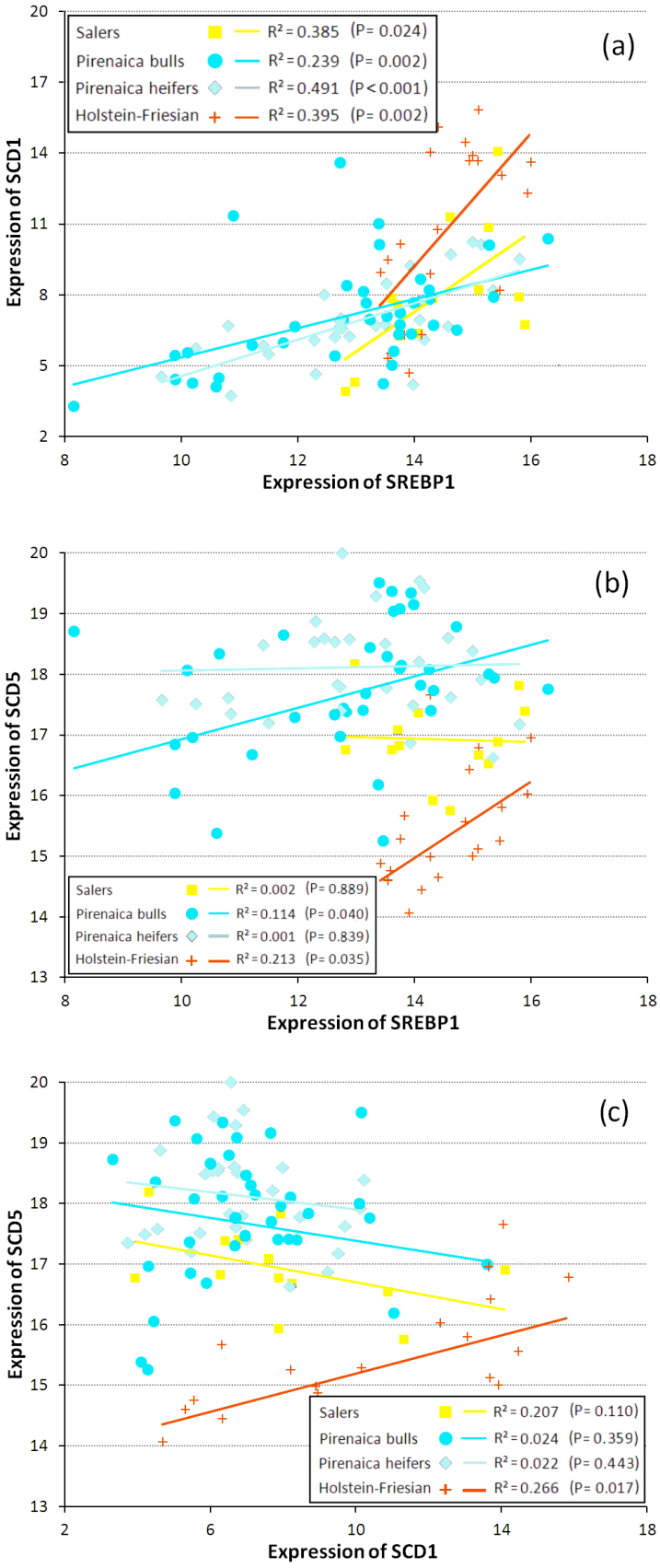


Figure IV.24. Estimated linear regression equations between (a) SCD1 and SREBP1, (b) SCD5 and SREBP1, and (c) SCD5 and SCD1.

On the other hand, the generally lower *SCD1* expression observed in Holstein-Friesian cows was previously reported in mature culled cows (Brooks et al., 2011), in which linoleic acid (18:2n-6) was suggested as the primary agent depressing *SCD* gene expression in adipose tissue (Smith et al., 2013). In **Figure IV.24.b**, significant correlations between *SCD5* and *SREBP1* were observed in Pirenaica bulls and Holstein-Friesian cows suggesting that *SCD5* expression may be more variable among commercial types than *SCD1* expression. This could be related to differences in the regulation of bovine *SCD5,* which was shown to be regulated by early growth response protein 2 (*EGR2*). *EGR2* and *SREBP1* appeared to bind to the same DNA binding site in the *SCD5* promoter, whereas other negative-regulation sites might also affect *SCD5* regulation (Lengi and Corl, 2012).

Partial Pearson correlations coefficients between gene expression (ΔCt) and desaturation index showed that *SREBP*1 expression was positively correlated with most of desaturation indexes in all studied commercial types (**Figure IV.25.a**). In general, Salers bulls showed the highest positive correlations (R > 0.65) between SCD1 expression and desaturation indexes for 9c-16:1, 9c-17:1, 9c-18:1, 9c-20:1, 7t,9c-18:2 and 9c,12t-18:2 (**Figure IV.25.b**). Pirenaica bulls also showed significant and positive correlations between *SCD*1 expression and desaturation indexes for 9*c*-17:1, 9*c*,13*t*-18:2, and 9*c*,15*c*-18:2 ($P < 0.05$). In contrast, few significant correlations between *SCD*5 and desaturation indexes among commercial types were observed (**Figure IV.25.c**). Surprisingly, a negative correlation between 9*c*-14:1 desaturation index and *SCD5* was observed in Pirenaica heifers ($P < 0.05$). Normally, feedstuffs are devoid of 9*c*-14:1 and, therefore, this FA is produced by *de novo* synthesis, suggesting that *SCD* gene expression may directly affect 9*c*-14:1 content.

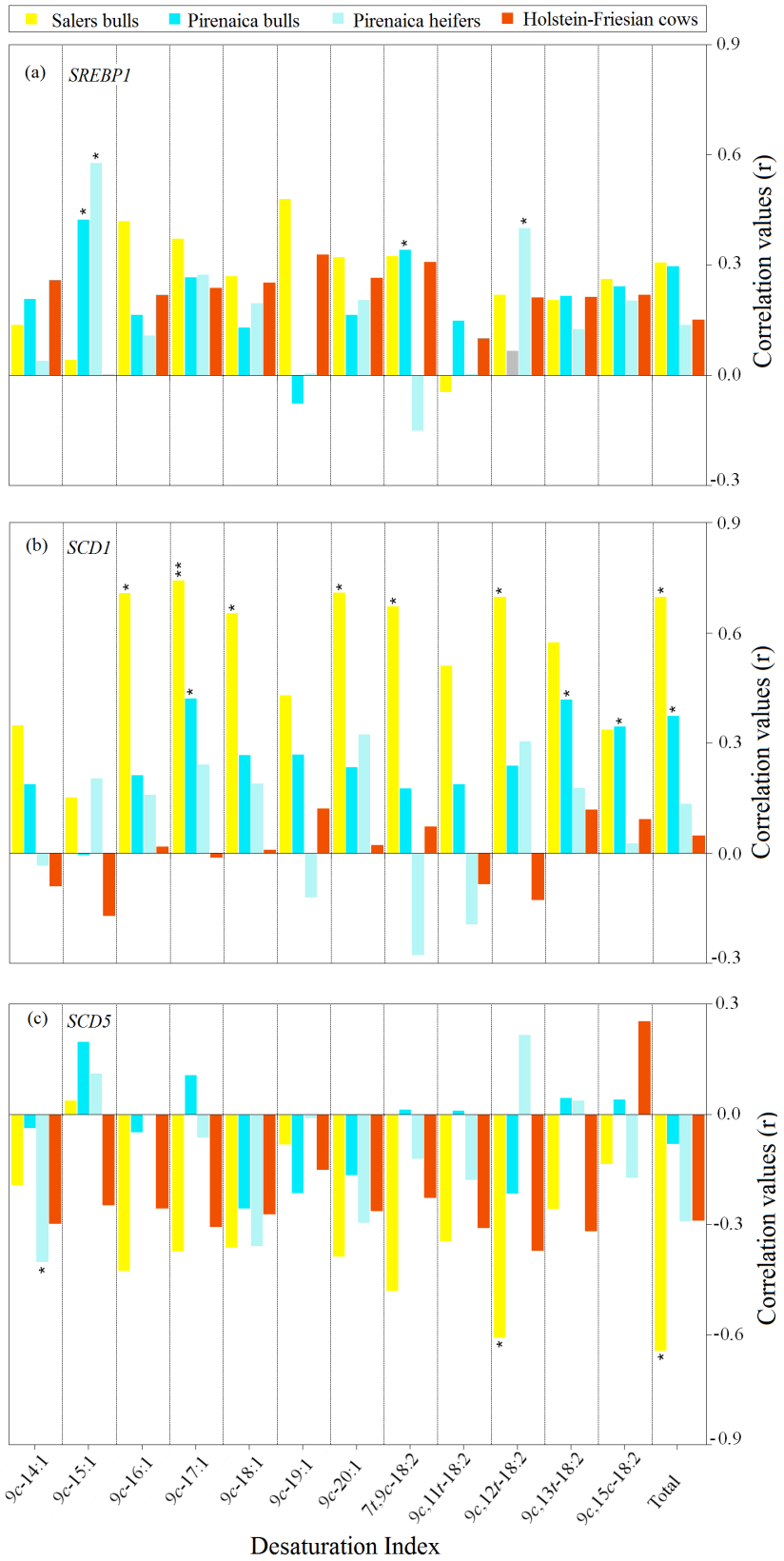


Figure IV.25. Partial correlations controlling for age and HCW between gene expression of *SREBP1* (a), *SCD1* (b), *SCD5* (c) and desaturation indexes calculated from fatty acid composition data of cattle commercial types. * $p < 0.05$, ** $p < 0.01$. Total is sum of all individual DIs.

In summary, these results revealed a potential opposite association between *SCD* isoforms in all breeds, suggesting that regulatory factors that upregulate *SCD*1 also downregulate *SCD*5 (and *vice versa*). However, both *SCD* isoforms are expressed in adipose tissue and may contribute to the maintenance of desaturation, being more than likely under a genetic compensation. Lower expression of one *SCD* isoform could well be compensated by the upregulation of the other isoform. This compensation theory was previously suggested in the model nematode *Caenorhabditis elegans* (Brock et al., 2011). The reciprocal expression observed between different isoforms and the underlying epigenetic processes require further investigation.

2.2. EFFECT OF A GENETIC POLYMORPHISM ON FATTY ACID COMPOSITION AND GENE EXPRESSION IN CATTLE

It is known that *SREBP1* gene plays a key role in the regulation of lipid biosynthesis, and therefore, any variant in the *SREBP1* sequence might affect its regulation and transcription. Moreover, *SREBP1* gene could be associated with the regulation of other lipogenic genes and lipid homeostasis. Several polymorphisms in *SREBP1* has previously related to FA composition (Hoashi et al., 2007; Rincon et al. 2012; Lee et al., 2013; Han et al., 2013), whereas the association of polymorphism with gene expression and FA composition has not been studied yet. In the present study a 84 bp insertion/deletion polymorphism (84 bp-indel; rs133958066) of intron 5 of *SREBP1,* has been characterized and its relationship with *SREBP1* gene expression and FA composition have been investigated (*Publication IV*).

The 84 bp-indel of bovine *SREBP1* gene showed two alleles (S and L) and 3 genotypes (SS, SL and LL), and the presence of S allele and SS genotype has been confirmed for the first time in Salers and Pirenaica cattle breeds by means of SSLP and Real-time PCR-HRM analysis (**Figure IV.26.**).

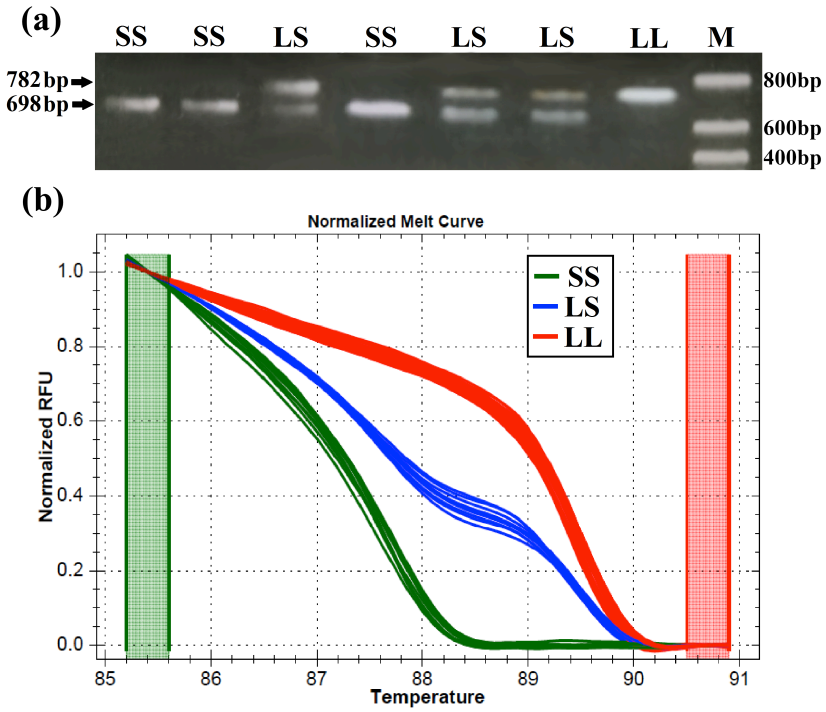


Figure IV.26. Genotyping of bovine SREBP1 gene. (a) Electrophoretic patterns of the DNA region containing the indel (S, 698 bp band; L, 782 bp band) and (b) normalized melting curves showing different 84bp-indel genotypes (HRM) of bovine SREBP1 gene. LL, Long homozygote; LS, Long-Small heterozygote; M, marker; SS, Small homozygote.

The maximum-likelihood mRNA tree of *SREBP1* showed a phylogenetic organization (**Figure IV.27.**) similar to what previously has been performed with mitochondrial DNA of ruminants and mammals (Hiendleder et al., 2008). The bovine sequence of *SREBP1* mRNA demonstrated high similarity with the sequence of other ruminants such as zebu (*Bos indicus*, 99%), buffalo (*Bubalus bubalis*, 85%), sheep (*Ovis aries*, 93%) and goat (*Capra hircus*, 96%). The alignment of intron 5 showed that similarity is higher in ruminants (99%–96%) compared with non-ruminant mammals (71%–45%; **Figure IV.27.b**). On the other hand, the similarity of *SREBP1* mRNA coding sequence, (**Figure IV.27.a**), was lower in comparison with the similarity of intron 5 (**Figure IV.27.b**) in ruminants.

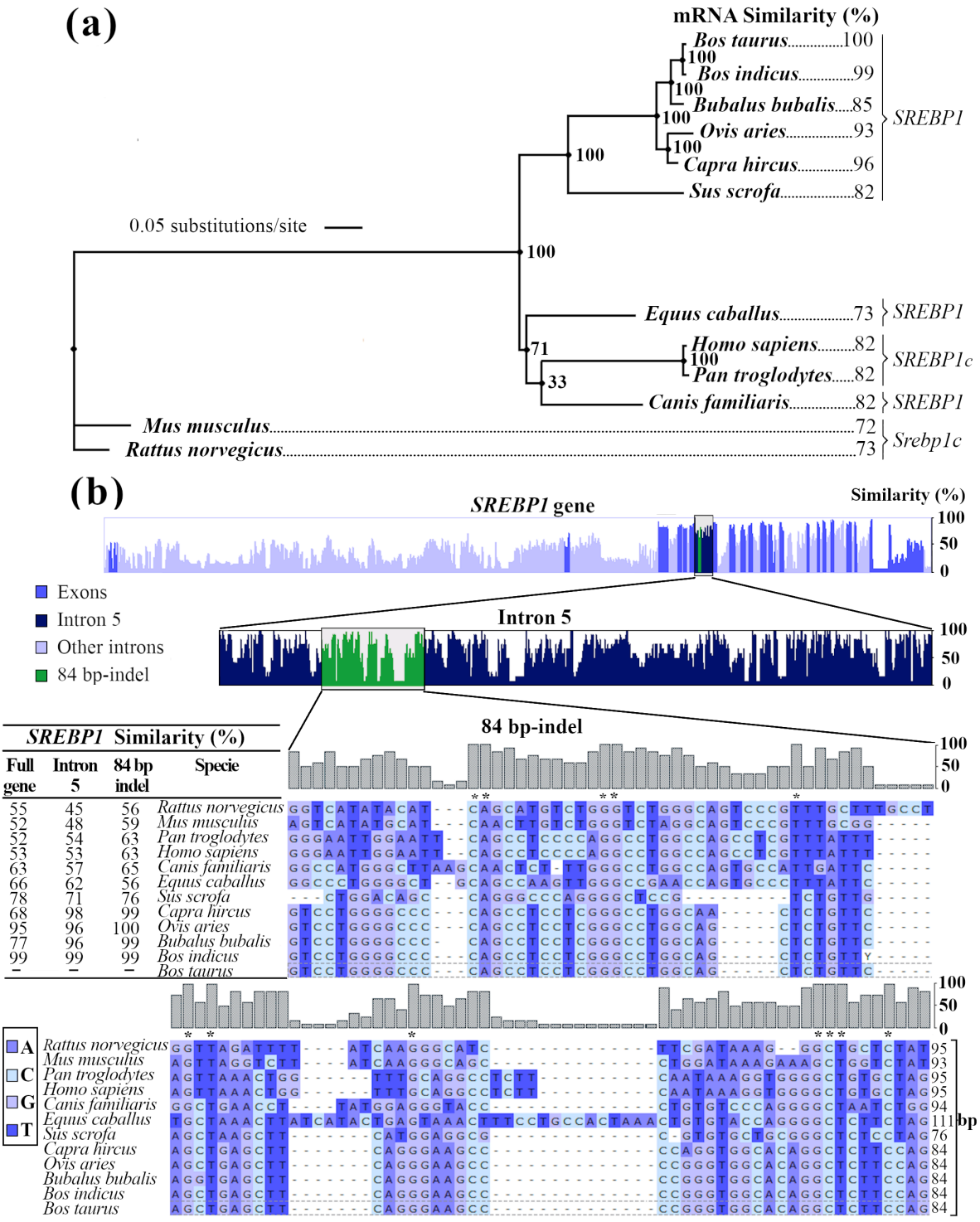


Figure IV.27. Representation of the nucleotide sequence similarities of *SREBP1* gene among mammals. (a) maximum-likelihood tree using mRNA of *SREBP1* in different mammals (the length of branches represents the expected number of substitutions per site) and the percentage of bootstrap replicates (up to 100) that support each node and the (b) area graphs (histograms) showing the similarity and the alignment of bovine genomic sequence of *SREBP1*, intron 5 and 84 bp-indel region along with homologous sequences of different mammals.

This result was unexpected since mRNA is normally translated to protein and usually shows high homology compared to non-coding introns. Therefore, the intron 5 could be a well-conserved region throughout evolution with an undetermined function in *SREBP1* and that could even regulate transcription levels of downstream genes. Accordingly, alignment analyses concluded that 84 bp-indel region seemed to be highly conserved in mammals. Moreover, the absence of S allele in mammals other than *Bos taurus* suggests that the 84 bp-indel polymorphism could have appeared after *Bos indicus* and *Bos taurus* species differentiation, reported to occur 1.7-2 million years ago (Hiendleder et al., 2008).

In terms of allele frequencies, the frequency of the minor allele (S) was higher in Salers bulls (0.385) compared to Pirenaica heifers (0.214) and bulls (0.135). In previous studies with bigger sample size, only SL and LL genotypes were reported in Simmental and crossbred breeds (Barton et al., 2010; Han et al., 2013). In contrast, as already reported, all Holstein-Friesian individuals showed single LL genotype (Huang et al., 2011; Kaneda et al., 2011; Proskura, 2013) which could have been the consequence of a high selection pressure towards milk production. Interestingly, and compared to the existing scientific literature, S allele frequency of Salers reached the highest values compared to other studied European beef cattle breeds.

In the present study, higher 18:0 content was observed in SS/SL compared to LL genotype in Pirenaica bulls ($p < 0.05$; **Table IV.10.**). In Pirenaica breed and in terms of PUFA content, the higher content in 18:3n-3 and total n-3 in SL compared to LL genotype may suggest an effect of this polymorphism on PUFA content ($p < 0.05$; **Table 4.11.**). These results were also corroborated with linear regressions in which Pirenaica breed (bulls and heifers together) and Pirenaica bulls alone showed significant linear regressions for 18:0, 18:3n-3 and n-3 contents (**Table IV.12.**). Interestingly, Bhuiyan et al. (2009) also reported higher muscle 18:2n-6 and total PUFA contents in SS compared to LL genotype in Hanwoo bulls.

Table IV.10. Effect of genotype (SS/SL vs LL) on the fatty acid content (mg/g fat) of subcutaneous adipose tissue of bovine commercial types (Salers bulls, Pirenaica bulls and heifers).

Fatty acid	Salers bulls			Pirenaica bulls			Pirenaica heifers			Holstein-Friesian cows
	SS/SL (n=7)	LL (n=6)	*p**	SS/SL (n=9)	LL (n=28)	*p**	SS/SL (n=9)	LL (n=19)	*p**	LL (n=21)
SFA	409 ± 23.7	365 ± 25.6	NS	388 ± 23.4	354 ± 13.3	NS	369 ± 12.9	377 ± 8.8	NS	406 ± 83.5
16:0	231 ± 9.8	208 ± 10.6	NS	216 ± 12.6	204 ± 7.10	NS	213 ± 8.1	228 ± 5.5	NS	228 ± 52.8
18:0	128 ± 12.11	117 ± 13.08	NS	131 ± 9.67	107 ± 5.48	0.037	111 ± 5.27	104 ± 3.61	NS	135 ± 60.25
MUFA	411 ± 12.1	414 ± 13.0	NS	373 ± 19.3	359 ± 11	NS	438 ± 11.3	439 ± 7.80	NS	356 ± 110
9*c*-16:1	32.1 ± 2.25	33.4 ± 2.43	NS	26.9 ± 2.68	29.9 ± 1.52	NS	32.7 ± 2.67	36.4 ± 1.83	NS	34.4 ± 24.47
9*c*-18:1	300 ± 8.84	304 ± 9.55	NS	269 ± 14.29	247 ± 8.1	NS	300 ± 11.83	303 ± 8.12	NS	261 ± 72.15
PUFA	30.2 ± 2.41	32 ± 2.61	NS	27.7 ± 2.50	24.5 ± 1.42	NS	24.4 ± 1.88	23.3 ± 1.29	NS	14.1 ± 4.21
n-6	28.0 ± 2.30	29.7 ± 2.49	NS	25.2 ± 2.37	22.4 ± 1.35	NS	22.1 ± 1.77	21.1 ± 1.22	NS	11.9 ± 3.89
18:2n-6	26.5 ± 2.29	28.3 ± 2.47	NS	24 ± 2.37	21.2 ± 1.35	NS	20.2 ± 1.72	19.4 ± 1.18	NS	10.6 ± 3.76
18:3n-6	0.102 ± 0.012	0.11 ± 0.013	NS	0.085 ± 0.018	0.13 ± 0.010	0.037	0.156 ± 0.018	0.165 ± 0.012	NS	0.141 ± 0.053
n-3	2.11 ± 0.134	2.17 ± 0.145	NS	2.47 ± 0.206	1.97 ± 0.117	0.042	2.23 ± 0.169	2.02 ± 0.116	NS	2.08 ± 0.623
18:3n-3	1.87 ± 0.133	1.9 ± 0.144	NS	2.21 ± 0.197	1.72 ± 0.111	0.040	1.91 ± 0.156	1.67 ± 0.107	NS	1.7 ± 0.583

The unit of fatty acid is denoted with mg/g of fat tissue. Values represented in this table are least square means ± standard deviations. *p** indicates *P*-values of the analysis of variance. The FA group descriptions (SFA, MUFA and PUFA) were referred to Publication IV.

Table IV.11. Effect of genotype (SS vs SL vs LL) on the fatty acid content (mg/g fat) of subcutaneous adipose tissue of all bovine commercial types† (Salers bulls, Pirenaica bulls and Pirenaica heifers) and Pirenaica breed‡ (bulls and heifers)

	All group (n=78) †				Pirenaica breed (n=65) ‡			
Fatty acid	**SS (n=7)**	**SL (n=18)**	**LL (n=53)**	***p****	**SS (n=4)**	**SL (n=14)**	**LL (n=47)**	***p****
SFA	363 ± 22.36	397 ± 13.9	363 ± 8.1	NS	334 ± 29.6	392 ± 15.6	363 ± 8.52	NS
16:0	208 ± 12.12	223 ± 7.53	213 ± 4.39	NS	190 ± 16.5	221 ± 8.71	214 ± 4.76	NS
18:0	112.3 ± 9.5^{a}	127 ± 5.91^{a}	106 ± 3.44^{b}	0.012	107 ± 12.2^{a}	126 ± 6.42^{a}	105 ± 3.51^{b}	0.022
MUFA	416 ± 18.06	402 ± 11.23	395 ± 6.54	NS	405 ± 24.6	399 ± 12.9	393 ± 7.09	NS
9*c*-16:1	32 ± 2.9	30 ± 1.81	33 ± 1.05	NS	30.2 ± 3.99	29.1 ± 2.11	32.7 ± 1.15	NS
9*c*-18:1	300.9 ± 15.01	282.7 ± 9.33	274.3 ± 5.44	NS	290 ± 20.1	278 ± 10.6	271 ± 5.79	NS
PUFA	26.36 ± 2.68	27.97 ± 1.66	24.77 ± 0.97	NS	23.7 ± 3.44	27.2 ± 1.82	23.9 ± 0.99	NS
n-6	24.16 ± 2.55	25.49 ± 1.59	22.65 ± 0.93	NS	21.4 ± 3.267	24.7 ± 1.724	21.8 ± 0.942	NS
18:2n-6	22.7 ± 2.524	24 ± 1.569	21.2 ± 0.915	NS	20 ± 3.236	23.2 ± 1.707	20.3 ± 0.933	NS
18:3n-6	0.106 ± 0.019	0.117 ± 0.012	0.141 ± 0.007	NS.	0.108 ± 0.027	0.12 ± 0.014	0.145 ± 0.008	NS
n-3	2.09 ± 0.205^{a}	2.37 ± 0.128^{a}	2.01 ± 0.074^{b}	0.050	2.13 ± 0.288^{a}	2.42 ± 0.152^{a}	1.99 ± 0.083^{b}	0.047
18:3n-3	1.83 ± 0.195^{a}	2.09 ± 0.121^{a}	1.72 ± 0.071^{b}	0.034	1.84 ± 0.272^{a}	2.15 ± 0.152^{a}	1.69 ± 0.078^{b}	0.028

† Corrected by breed and sex. ‡ Corrected by sex. *p** indicates *P*-values of the analysis of variance. Significant difference is defined as $p < 0.05$, otherwise it is denoted as NS (not significant) or NS. (Trend, $p < 0.1$). The unit of fatty acid is denoted with mg/g of fat tissue; The values represented in this table are least square means ± standard deviations; The FA group descriptions (SFA, MUFA and PUFA) were referred to Publication IV.

Table IV.12. Regression equations between the genotype (SS/SL vs LL) and the fatty acid content (mg/g fat) of subcutaneous adipose tissue of Pirenaica (bulls & heifers), Pirenaica bulls and Pirenaica heifers

Fatty acid	Pirenaica bulls & heifers			Pirenaica bulls			Pirenaica heifers		
	Slope	SEM	*p*	Slope	SEM	*p*	Slope	SEM	*p*
18:0	-15.8	6.740	0.022	-24.2	10.9	0.034	-6.81	6.41	NS
n-6	-2.04	1.788	NS	-2.81	2.705	NS	-0.948	2.16	NS
18:2n-6	-1.96	1.784	NS	-2.87	2.702	NS	-0.849	2.10	NS
18:3n-6	0.023	0.016	NS	0.045	0.021	0.036	0.008	0.022	NS
n-3	-0.371	0.156	0.020	-0.504	0.234	0.038	-0.202	0.206	NS
18:3n-3	-0.377	0.149	0.015	-0.488	0.224	0.036	-0.235	0.189	NS

*p indicates p-values of the analysis of variance. Significant difference is defined as $p < 0.05$, otherwise it is denoted as NS (not significant). SEM, Standard error of the mean.

Even 84 bp-indel has been associated with several FAs (**Table IV.10.**), *SREBP1* and *SCDs* gene expressions did not differ among 84 bp-indel genotypes in Pirenaica or other breeds (*Publication V*). Thus, further analysis in the *SREBP1* promoter might help to clarify the underlying mechanism of 84-bp indel and its relationship with FA composition. The association of other *SREBP1* polymorphisms with FA composition have also been reported in the literature (Lee et al., 2013), and, therefore linkage disequilibrium with other causative polymorphisms could also explain the FA differences.

On the other hand, Holstein-Friesian, dairy culled cows, demonstrated a significant correlation between *SREBP1* expression and 16:0 and MUFA including 9*c*-16:1 and 9*c*-18:1 FA contents ($p < 0.01$), whereas not significant correlations were observed in Pirenaica and Salers, primarily oriented to meat production (**Figure IV.28.**).

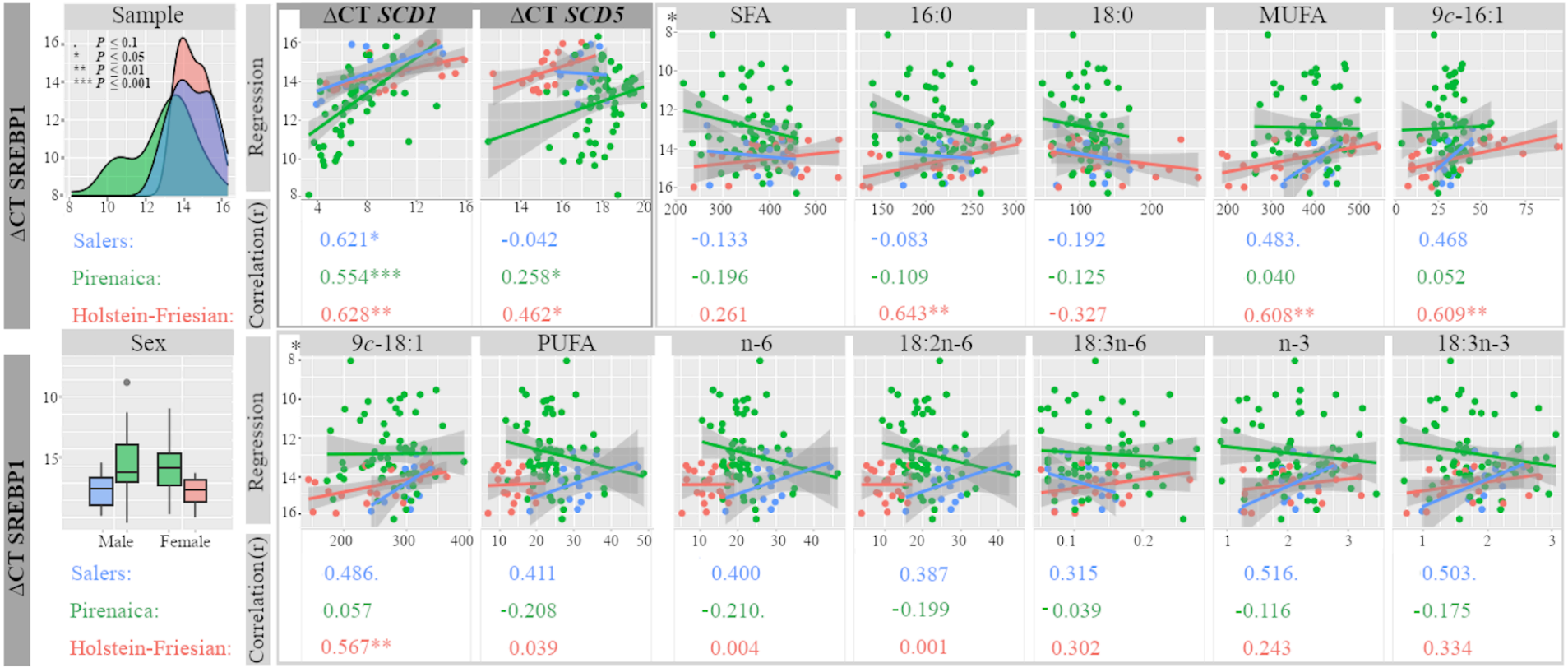
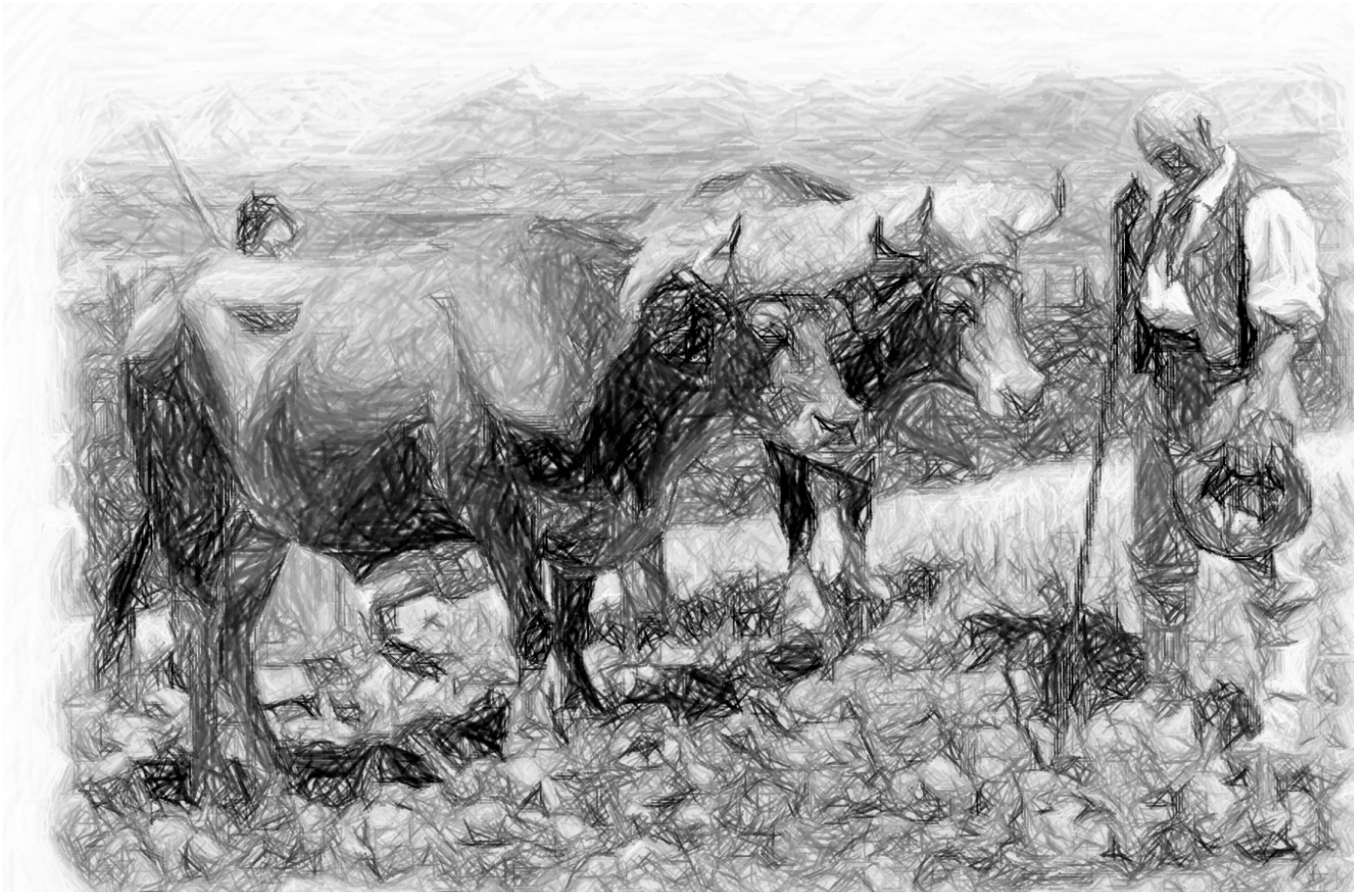


Figure IV.28. Regression and correlations between lipogenic gene expression and FA content of bovine commercial types. Gene expression level and FA contents are represented with ΔCT and mg/g of fat tissue, respectively. *The scale of ΔCT *SREBP1* is inverted to show direct correlation between *SREBP1* gene expression and FA content. * is defined as $P < 0.05$, ** as $P < 0.01$, *** as $P < 0.001$ and "." as a Trend, $P < 0.1$.

In a previous section (*Section 4.2.1.*), a novel genetic compensation mechanism was reported between *SCD1* and *SCD5* that showed that one *SCD* isoform could well be compensated by upregulation of the other isoform. This opposite pattern has been consistently detected regardless of the 84 bp-indel genotype (*Publication V*). As such, significant correlations between *SCD1* and SFAs (including 16:0) were observed in SL genotype of Pirenaica bulls, but also 9*c*-16:1 when all Pirenaica individuals were considered. On the other hand, *SCD5* was negatively correlated with 16:0 ($p < 0.05$). As it has been described in the scientific literature, several transcription factors apart from *SREBP1* such as *liver X receptor* (*LXR*), *peroxisome proliferator-activated receptor alpha* (*PPARα*), *CCAAT/enhancer binding protein alpha* (*C/EBPα*), *nuclear factor-1* (*NF-1*), *nuclear factor-Y* (*NF-Y*), and *Sp1 transcriptional factor* (*Sp1*) could have been responsible for binding the *SCD* promoter region (Mauvoisin & Mounier, 2011), and therefore, they could have also been involved in the regulation of *SCD1*/*SCD5* compensation pattern.

V. REFERENCES



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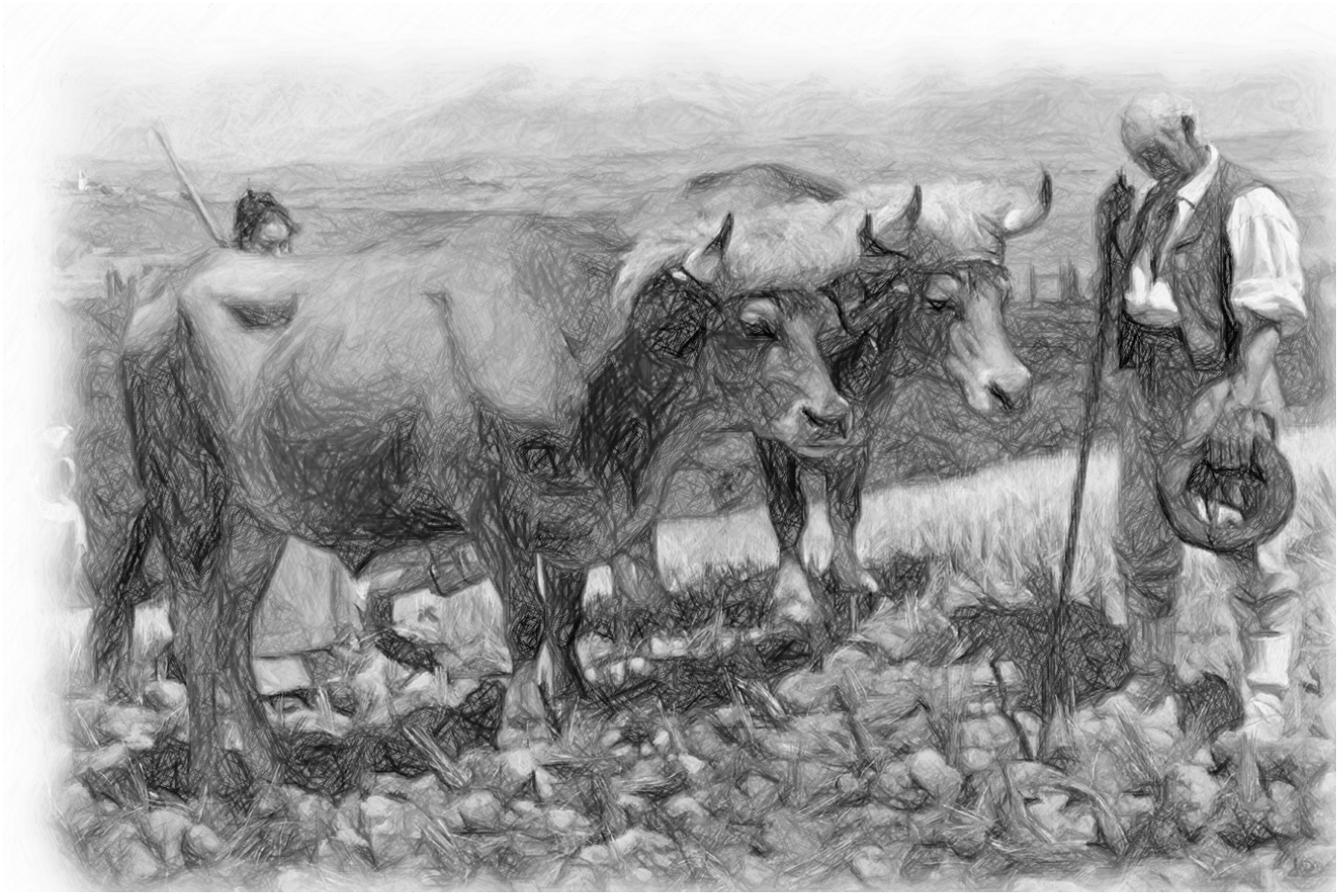
SECTION II.

CONCLUSIONS



Based on the experimental results obtained in the present Ph. D. Thesis, the following conclusions were drawn:

1. The use of a commercial multiplex kit containing 12-STR markers facilitated the study of preliminary diversity parameters in cattle, and demonstrated the efficacy for identification and parentage purposes. However, one locus (TGLA53) showed an inaccurate genotyping performance, and therefore, even though this commercial STR panel revealed a sufficient discrimination power for case identification, an increase in the loci number is recommended for a higher power of exclusion in paternity studies.

2. The genetic characterization of allochthonous Salers cattle breed revealed the presence of African zebu diagnostic alleles, supporting the hypothesis of a migration route through the Mediterranean region and the presence of a genetic signature characteristic of North African cattle in this breed.

3. The heterozygosity of allochthonous Salers revealed the absence of a founder effect in its expansion in the Basque Country and Spain. In addition, Spanish Salers population seemed to be under mutation-drift equilibrium which indicates a constant incorporation of breeding animals to the population.

4. Salers genetic structure and its relationship with other European breeds revealed a significant and a well-defined genetic differentiation, being genetically closer to Blonde d´Aquitaine, Limousin and Charolais breeds compared to other more distant European cattle breeds.

5. The genetic characterization of autochthonous Pirenaica breed revealed the presence of African zebu diagnostic alleles, indicating a North African genetic signature and, therefore, supporting the Neolithic migration from Africa to the Mediterranean area extended to the Basque region.

6. The study of several STR marker sets demonstrated the effectiveness of 30-STRs for parentage in autochthonous Pirenaica breed and for traceability when investigated against other closely related local breeds. A minimum of a 21-STR panel is necessary

for a reliable parentage study in the absence of genetic information from one parent and for trustworthy assignments based in machine-learning predictive models.

7. The differences in fatty acid composition of adipose tissue among several bovine commercial types (Salers bulls, Pirenaica bulls, Pirenaica heifers and Holstein-Friesian cows) were associated to genetic variability in lipogenic gene expressions. The effect of gene expression of lipogenic genes on the desaturation and fatty acid composition seemed to be influenced by breed and gender.

8. The gene expression of *SCD1* was demonstrated to be dependent on *SREBP1* gene expression due to the strong correlations between them independently of the commercial type studied. Furthermore, the opposite correlations between *SCD1* and *SCD5* isoforms suggested a novel genetic compensatory regulation of the SCD activity.

9. The 84 bp-indel polymorphism has been described for the first time in the intron 5 of *SREPB1* gene of Salers and Pirenaica cattle breeds. Within ruminants, the non-coding intron 5 of *SREBP1* showed higher similarity compared to other introns of the same gene, which suggests that this is a region well-conserved throughout evolution.

10. The 84 bp-indel polymorphism demonstrated to be associated with the fatty acid composition among studied bovine commercial types, especially in Pirenaica breed, which S allele and SL genotype were associated with higher saturated (18:0) and polyunsaturated (18:3n-3) fatty acid contents of subcutaneous adipose tissue. The 84 bp-indel region may have a function in *SREBP1* or even act regulating transcription levels of downstream genes.

SECTION III.

APPENDIX I.

PUBLICATION I.

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Forensic efficacy of twelve STRs in Spanish cattle

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ABSTRACT

Cattle (*Bos taurus*) is an important meat food source for humans. STR loci have been extensively used for parentage testing in high demand by the animal breeding industry, but have also shown useful for forensic purposes to carry out identification of stolen animals or ones involved in traffic accidents. The International Panel of Microsatellites for Cattle Parentage Testing established 12 microsatellites for cattle parentage analysis (ISAG 2008). At present, there are few commercial kits for meat traceability and cattle parentage testing. In this study, based on a data set of 431 animals, an assessment of the Finnzymes Bovine Genotype™ panel with 12 STRs (BM1818, BM1824, BM2113, ETH10, ETH225, ETH3, INRA023, SPS115, TGLA122, TGLA126, TGLA227, and TGLA53) is reported for forensic investigations. Genotypes from STR loci were used to calculate the power of exclusion, matching probability, power of discrimination, and null allele frequencies and check the power of these loci as a minimum standard for identity and kinship analysis.

1. Introduction

Animal forensic genetics is being implemented as a relevant subject for legal matters concerning animal traces. Forensic investigations have shown to be useful in cases that involve animals as victims, (cruelty or poaching), suspects (animal attacks, accidents, or property damage), or witnesses (biological sample from an animal related to a human suspect or victim). Microsatellite (STR) loci have been revealed useful for parentage testing in high demand by the animal breeding industry, but also for other forensic purposes. As an important source of food in the human diet, forensically relevant cases involving cattle, such as identity forgery or cattle rustling, have been usual [1–3].

The International Panel of Microsatellites for Cattle Parentage Testing (ISAG Panel) established recommendations including nine microsatellite and later three additional markers as candidate loci in cattle parentage analysis (ISAG 2008). At present, there are few commercial reagent kits for meat traceability and cattle parentage testing. In this study, the assessment of the efficacy in a commercial kit of 12 bovine STRs is reported for forensic investigations.

2. Material and methods

Blood samples of 431 cattle individuals (403 Salers breed and 28 Terreña breed) were collected. Genomic DNA was extracted with the Gentra PureGene Blood Kit (Qiagen, Valencia, CA, USA). The microsatellites studied were those included in the international selected set of markers recommended by the FAO and ISAG for cattle diversity studies. Multiplex PCR reaction was performed using the commercial Bovine Genotypes™ Panel 1.2 (Finnzymes Diagnostics, Espoo, Finland) that includes 12 STRs: BM1818, BM1824, BM2113, ETH10, ETH225, ETH3, INRA023, SPS115, TGLA122, TGLA126, TGLA227, and TGLA53. Capillary electrophoresis was performed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) using internal size standard GS LIZ500 (Applied Biosystems). Fragment analysis was determined by GeneMapper v4.0.

CERVUS v3.0.3 software was used to calculate the number of alleles per locus (k), null allele frequency (F (null)), probability of exclusion in duos (PE1), probability of exclusion in trios (PE2), HO, and HE for both populations. The combined power of exclusion was calculated with the formula $CPE = 1 - (1 - PE1)(1 - PE2)(1 - PE3)(1 - Pk)$. The polymorphism information content (PIC), power of

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Table 1
K: number of alleles per locus, HO: observed homozygosity, HE: expected heterozygosity, PI: paternity index, PIC: polymorphic information content, PD: power of discrimination; PE1: power of exclusion for duo, PE2: power of exclusion for trio, CPE1: combined power of exclusion for duo, CPE2: combined power of exclusion for trio, HW: Hardy–Weinberg equilibrium, *F* (null): null allele frequency.

Breed	Marker	*K*	HO	HE	PI	PIC	PD	PE1	PE2	CPE1	CPE2	HW	*F* (null)
TERREÑA	BM1818	6	0.643	0.523	1.40	0.490	0.7704	0.15	0.322	0.150	0.322	0.942	−0.163
	BM1824	4	0.750	0.744	2.00	0.681	0.8647	0.307	0.481	0.411	0.648	0.745	−0.010
	BM2113	10	0.929	0.869	7.00	0.837	0.9260	0.544	0.707	0.731	0.897	0.336	−0.042
	ETH10	3	0.536	0.458	1.08	0.386	0.6199	0.101	0.215	0.758	0.919	0.561	−0.104
	ETH225	5	0.643	0.766	1.40	0.708	0.8826	0.340	0.516	0.841	0.961	0.128	+0.080
	ETH3	7	0.821	0.755	2.80	0.699	0.8546	0.337	0.513	0.894	0.981	0.380	−0.051
	INRA023	10	1.000	0.867	14.00	0.834	0.9286	0.537	0.701	0.951	0.994	0.614	−0.082
	SPS115	8	0.786	0.749	2.33	0.708	0.8954	0.354	0.538	0.968	0.997	0.912	−0.034
	TGLA122	6	0.536	0.729	1.08	0.663	0.8724	0.294	0.463	0.978	0.998	0.154	+0.146
	TGLA126	5	0.714	0.697	1.75	0.630	0.8087	0.268	0.434	0.984	0.999	0.228	−0.032
	TGLA227	9	0.857	0.846	3.50	0.811	0.9337	0.498	0.669	0.9918	0.9997	0.936	−0.017
SALERS	BM1818	7	0.340	0.324	0.76	0.313	0.5514	0.057	0.187	0.057	0.187	0.831	−0.059
	BM1824	5	0.720	0.729	1.78	0.680	0.8794	0.311	0.485	0.350	0.581	0.231	+0.006
	BM2113	9	0.730	0.710	1.85	0.682	0.8870	0.323	0.51	0.560	0.795	0.321	−0.011
	ETH10	7	0.744	0.714	1.96	0.665	0.8642	0.300	0.472	0.692	0.892	0.784	−0.013
	ETH225	5	0.772	0.770	2.19	0.731	0.9064	0.369	0.547	0.806	0.951	0.116	+0.004
	ETH3	8	0.576	0.583	1.18	0.530	0.7741	0.183	0.339	0.841	0.967	0.230	+0.011
	INRA023	8	0.695	0.731	1.64	0.698	0.8920	0.342	0.524	0.895	0.984	0.013	+0.020
	SPS115	6	0.201	0.211	0.63	0.203	0.3595	0.023	0.112	0.898	0.986	0.013	+0.062
	TGLA122	9	0.687	0.696	1.60	0.659	0.8732	0.295	0.476	0.928	0.993	0.516	+0.016
	TGLA126	5	0.588	0.590	1.21	0.503	0.7477	0.176	0.300	0.941	0.995	0.099	+0.001
	TGLA227	12	0.742	0.769	1.94	0.741	0.9097	0.396	0.576	0.9642	0.9979	0.000	+0.022

discrimination (PD), and paternity index (PI) were calculated with PowerStats v1.2 (Promega, Madison, WI, USA). Exact tests for deviations from the Hardy–Weinberg equilibrium (HW) were performed with GENEPOP v.4.2.

3. Results and discussion

Twelve loci microsatellites were analyzed but the TGLA53 microsatellite did not render results caused by inhibition during amplification. The genotype determination was doubtful due to three stutters per allele and the unbalanced size of alleles. The marker was excluded from analysis because of the low and uncertain efficiency of genotype determination as previously described by other authors [4,5].

All the studied loci were polymorphic and 86 alleles were detected. The number of alleles per locus ranged between 3 (ETH10) and 12 (TGLA227), with an average of 6.64 in Terreña and 7.36 in Salers, as shown in Table 1.

The HW equilibrium was respected in all markers, except for TGLA227 in Salers even when the Bonferroni correction was applied. The TGLA227 heterozygote deficit was only found in a specific Salers herd, which may be due to certain reproductive isolation or inbreeding, although it was not extended to the total Salers sample. The average expected heterozygosity was 0.846 in Terreña and an excess of heterozygotes was also observed. While in Salers, the average expected heterozygosity was 0.769 and a deficit of heterozygotes was revealed. In mean values, Terreña showed higher heterozygosity. The PIC values recorded per locus varied between 38.6% and 83.7% for Terreña and between 20.3% and 74.1% for Salers, with mean values of 66.7% and 58.2%, respectively. Although the Terreña sample was smaller than that of Salers, the Terreña breed reflects slightly greater genetic variability, probably due to local use and management in comparison with the commercial selection used in Salers. According to Botstein et al. [6], values below 50% can be considered uninformative, although in the present case, this can only be applied to BM1818 (49%) and ETH10 (38.9%) in Terreña, and BM1818 (31.3%) and SPS115 (20.3%) in Salers.

The forensic efficiency evaluation for 11 loci of this commercial multiplex kit showed higher values of the combined power of discrimination in Terreña ($CPD \geq 0.9999999998$) than in Salers ($CPD \geq 0.999999996$). The power of exclusion per locus ranged between 0.101 (ETH10) to 0.544 (BM2113) in duos (PE1) in the Terreña breed, while it ranged between 0.023 (SPS115) to 0.396 (TGLA227) in Salers. Higher values were observed in trios (PE2), ranging from 0.215 (ETH10) to 0.707 (BM2113) in Terreña, and 0.112 (SPS115) to 0.576 (TGLA227) in Salers. In addition, the combined paternity of exclusion probabilities was ($CPE1 \geq 0.9918$) in duos and ($CPE2 \geq 0.9997$) in trios for Terreña, whereas it was ($CPE1 \geq 0.9642$) in duos and ($CPE2 \geq 0.9979$) in trios for Salers, proving the discriminatory strength of this panel. CPE values are directly related to the number of loci analyzed, so these results are consistent with the fact that this analysis included eleven loci.

Taking into account that this multiplex consists of 12 loci but that one locus was excluded, the 12 loci recognized by ISAG and available in the present commercial kit reveal that the number of STRs should be increased to reach a higher power of exclusion. Nine loci were recommended by ISAG as the minimum set in 1990 and it was increased with three additional markers in 2008. This selection was made with the consensus agreement after several interlaboratory comparison tests involving several rounds. Today, although these loci have been expansively used and the creation of many databases justifies their maintenance, the review of the presence of the ineffective TGLA53 in the present commercial kit is suggested. In addition, even if some of these loci have showed low polymorphism in present breeds, it is not practical to substitute any of these loci, and it would be more convenient to increase the number of loci to achieve statistical power suitable for forensic applications.

4. Conclusion

This study describes a forensically useful discriminative multiplex that permits the analysis of 12 STRs with a commercial kit. Nevertheless, increasing the number of loci is suggested to

provide a higher power of discrimination and power of exclusion for paternity testing.

Conflict of interest

The authors declare that they have no conflict of interests.

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APPENDIX II.

PUBLICATION II.

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Genetic characterization and founder effect analysis of recently introduced Salers cattle breed population

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Salers are a native French breed used for beef and dairy production that has expanded to all the continents. The Salers breed was introduced to the north of Spain in 1985 with only 15 individuals from France and has successfully increased to over 20 000 animals. Although over time new animals have been imported from France for breeding, it is possible that the limiting number of founder animals could have resulted in a reduction of the genetic diversity found in Spanish Salers. Thus, the purpose of the present study has been to characterize the genetic diversity of Salers breed in Spain and evaluate a possible founder effect due to reduced number of the first reproducers. A total of 403 individuals from 12 Salers herds were analyzed using 12 microsatellite markers and compared with phylogenetically and geographically close related Blonde d'Aquitaine, Limousin and Charolais French breeds but also other 16 European breeds. Microsatellites in Salers were polymorphic, with a mean allelic richness of 5.129 and an expected heterozygosity of 0.621 across loci (0.576 to 0.736 among all breeds). Average observed heterozygosity was 0.618. All the loci fit the Hardy–Weinberg (HW) equilibrium except TGLA227 locus due to a significant deficit of heterozygotes in only one of the herds, probably attributable to a sampling effect. When all loci were combined, Salers inbreeding coefficient did not differ statistically from 0 ($F_{IS} = 0.005$), indicating not significant excess or deficit of heterozygotes ($P = 0.309$). Based in allelic distribution, Salers revealed a frequency of 0.488 in BM2113-131 and 0.064 in BM2113-143 diagnostic alleles, which are specific to the African zebu. These zebu alleles are also found in some French breeds, supported by STR data previously postulated hypothesis of a migration route through Mediterranean route by which North African cattle may have left a genetic signature in southern Europe. Phylogenetic tree and population structure analyses could unambiguously differentiate Salers cattle from the other populations and 10% of the total genetic variability could be attributed to differences among breeds (mean $R_{ST} = 0.105$; $P < 0.01$). Mutation-drift equilibrium tests (sign test and Wilcoxon's sign rank test) were in correspondence to the absence of founder effect when Bonferroni was applied. Gene diversity previously reported in French Salers was comparable with the observed in our population. Thus, high genetic diversity in Spanish Salers highlights the resources of this population, which looks toward future breeding and selection programs.

Keywords: admixture, founder effect, genetic structure, microsatellite, subdivided populations

Implications

This study presents the genetic diversity of Salers breed in Spain originated from a very low small number of individuals and evaluates if a loss of genetic variation could be occurred. In general, our results could have shown high genetic diversity in Spanish Salers as a first step toward signaling the adequacy of this population for breeding and selection strategies.

In addition, Spanish Salers cattle have shown genetic markers representative of African zebu cattle breeds. These markers were also found in other breeds from France and therefore this results support the previously postulated theory of an African heritage in southern European breeds.

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Introduction

Salers are a French native and dual-purpose (beef/dairy) breed which originates from the high Massif Central in the Auvergne region of France. It is a rustic breed used for beef production in mountainous areas of central France that shows excellent maternal aptitudes, hardiness and satisfactory milk yield (Petit and Liénard, 1988) whose milk is also traditionally used to produce French (AOC) Controlled Designation of Origin cheese called Salers.

Salers breed dates from the middle of the 19th century and its Herd Book was created in 1906. Since then, Salers have been widely spread in France and to nearly 30 countries on five continents (Salers Evolution Group, 1992). Salers breed was first introduced to the north of Spain in 1985 with only

15 individuals from France. The population has grown to ~22 000 individuals mainly focused in the northern Iberian Peninsula. Although afterwards, more animals were imported from France for breeding, a founder effect could have occurred as a consequence of the low number of first reproducers. Genetic diversity is thought to decrease due to founder effects (Böhme *et al.*, 2007) and theoretically, we could expect changes in allele frequencies due to genetic drift. In present case, Spanish Salers genetic characterization is needed to evaluate whether a founder effect has occurred, or the importation of relatively few animals and good breeding management has been enough to maintain mutation-drift equilibrium. This data could be interesting in the idea of future selection programs for economic animal improvement in Spanish Salers.

The genetic characterization of the Salers breed and its relationships to other European cattle breeds has previously been studied by different approaches. Grosclaude *et al.* (1990) and Blott *et al.* (1998) analyzed polymorphisms from blood groups, milk proteins and serum to discriminate between cattle breeds, Salers included. Microsatellite loci are more variable than biochemical markers, thus permit fine-scale analysis of genetic structure and the precise measurement of genetic variation (Moazami-Goudarzi *et al.*, 1997). Even so, few Salers genetic studies have been published so far. Three studies have included genetic diversity of French Salers by using microsatellite loci in few samples (Canon *et al.*, 2001; Cymbron *et al.*, 2005; Amigues *et al.*, 2011) but exhaustive diversity genetic data of Spanish Salers have not yet been reported and preliminary parentage testing data has only been published (Gamarra *et al.*, 2015). Thus, this study centers itself on the evaluation of a founder effect in Salers population that could describe the breeding management used in Spain for a recently introduced breed. In addition, it contributes to the knowledge of the genetic relationships between Spanish Salers and other European breeds.

Material and methods

Sample collection and DNA extraction

Blood samples of 403 Salers cattle breed individuals from 12 different herds were analyzed. Bovine genomic DNA was extracted from blood using salting out extraction with Gentra PureGene Blood kit (Qiagen, Valencia, CA, USA) following the protocol handbook. The DNA samples were stored at −20°C before use.

Microsatellite amplification and analysis

The microsatellites used are included in the international selected set of markers recommended by Food and Agriculture Organization and International Society of Animal Genetics for cattle diversity studies. Amplification of microsatellites was performed by multiplex PCR reaction using commercial Bovine Genotypes™ Panel 3.1 (Finnzymes Diagnostics, Espoo, Finland) according to the manufacturer's instructions. Capillary electrophoresis was performed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using internal size standard GS LIZ500 (Applied Biosystems). Fragment analysis was determined by GeneMapper v4.0. Alleles were coded according to reference samples.

TGLA53 marker showed high inhibition during the amplification. Thus, TGLA53 microsatellite was sequenced in 10 samples of uncertain genotype to identify any possible single nucleotide polymorphism (SNP) in flanking regions. It was amplified with forward 5′ACTCTATTGACCATTGTAGGCTC3′ and reverse 5′TTATAGCATTTATCTCCCACCCTC3′ primers to a concentration of 0.1 μM, $MgCl_2$ at 2.5 mM, buffer 1×, and dNTPs at 0.2 mM, in a C1000TM Thermal Cycler (Bio-Rad, Foster City, CA, USA) at 95°C for 3 min; 30 cycles at 95°C for 30 s, 64°C for 30 s and 72°C for 90 s; final extension at 72°C for 7 min. These products were purified by vacuum ultrafiltration using MultiScreen® PCRμ96 plates (Millipore, Billerica, MA, USA) and sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI3130 Genetic Analyzer (Applied Biosystems) using the Sequencing Analysis v5.2. All the sequences have been deposited into GenBank under accession numbers KP874721-KP874730.

Statistical analysis

GENEPOP 4.2 software (Raymond and Rousset, 1995) was used to test for deviations from HW equilibrium using exact test of Guo and Thompson (1992). The program performed a probability test using a Markov chain (dememorization 5000, batches 100, iterations per batch 1000). Significant levels were calculated per locus and per breed. Bonferroni's procedures were applied when necessary.

The FSTAT 2.9.3 software (Goudet, 1995) was used to calculate the number of alleles per locus (Na), allelic richness and inbreeding coefficient (F_{IS}) per locus and the *F*-statistics (Weir and Cockerham, 1984) estimates in all breeds. Expected (He) and observed (Ho) heterozygosities and polymorphism information content were calculated with Cervus 3.0 (Kalinowski *et al.*, 2007).

BOTTLENECK (Piry *et al.*, 1999) was used to study a possible founder effect by determining a significant number of loci with heterozygosity excess. Sign test and Wilcoxon's sign rank test (Cornuet and Luikart, 1996) were performed under stepwise mutation model (SMM) and two-phase model (TPM; 95% SMM with 5% multi-step mutations and a variance among multiple steps of 12) which is considered best for microsatellite data (Di Rienzo *et al.*, 1994; Piry *et al.*, 1999). In addition, a qualitative descriptor of the allele frequency distribution (mode-shift indicator) which discriminates bottlenecked populations from stable populations (Luikart *et al.*, 1998) was used.

The Spanish Salers were analyzed in comparison with 20 breeds previously described by Van de Goor *et al.* (2011), shown in Table 1. Reynolds genetic distance measures were computed by Arlequin 3.5 (Excoffier and Lischer, 2010) and a neighbor-joining (NJ) tree was constructed. TreeFit (Kalinowski, 2009) was used to create NJ tree and calculate R^2 to quantify the degree to which bifurcating trees distort

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Table 1 *Origin, abbreviation and number of samples (n) of Spanish Salers and other breeds used for comparison included in this study*

Origin	Breed	Abbreviation	*n*
France	Salers	SAL	403
	Blonde d'Aquitaine	BLA	165
	Limousin	LIM	126
	Charolais	CHL	28
Ireland	Dexter	DEX	428
Great Britain	Scottisch Highlander	SHL	118
	Hereford	HER	62
	Galloway	GAL	88
Belgium	Belgian Blue	BBL	51
Germany	Heck Cattle	HEC	39
Austria	Waldviertler Blondvieh	WAL	45
Italy	Marchigiana	MAR	17
The Netherlands	Holstein Friesian	HFR	2507
	Holstein	HOL	254
	Dutch Friesian	DFH	42
	Groningen Whiteheaded	GWH	24
	Dutch Belted	DLV	24
	Maas Rijn Ijssel	MRY	41
	Verbeterd Roodbont	VRB	42
	Brandrood Cattle	BRR	41
Japan	Wagyu	WAG	20

genetic relationships between populations. Genetic differentiation among samples was estimated using R_{ST} (Rousset, 1996) with FSTAT v.2.9.3.

The existence of distinct genetic groups using multilocus genotypes was tested using a Bayesian model-based clustering method in STRUCTURE 2.3.4 (Pritchard *et al.*, 2000). The probability of an admixture model was tested for clusters (*K*) ranging from 1 to 21. Models were tested using 20 independent Markov chain Monte Carlo (MCMC) simulations, each consisting of 1×10^5 iterations after a burn-in of 5×10^5 iterations. The optimal value of *K* was selected using the approach ΔK of Evanno *et al.* (2005) and the web interface Structure Harvester (Earl and VonHoldt, 2012). An animal was attributed to a given cluster when the proportion of its genotype in the cluster (q_K) was higher than an arbitrary cutoff value of 80%, the same threshold employed in previous studies (Vigouroux *et al.*, 2008). A structure graphical bar plot of membership coefficients for populations and individuals was generated using the Distruct program (Rosenberg, 2004). A correspondence analysis (CA) was used in conjunction with STRUCTURE 2.3.4. This multivariate method leads to a simultaneous representation of breeds and loci as a cloud of points in a metric space. Genetix 4.05.2 (Belkhir *et al.*, 2004) was used to represent the three-dimensional plot.

Results

Genetic characterization of Spanish Salers breed

A total of 12 microsatellite loci (BM1818, BM1824, BM2113, ETH10, ETH225, ETH3, INRA023, SPS115, TGLA122, TGLA126, TGLA227 and TGLA53) were analyzed in 403 Spanish Salers individuals. All the loci were polymorphic and 81 alleles were detected. Number of alleles per locus ranged between five (BM1824, ETH225 and TGLA126) and 12 (TGLA227) with an average of 7.36 as shown in Table 2. Rare alleles, defined as those with frequencies below 5%, were observed and remarkable non-European alleles were found in some microsatellite markers. The African zebu BM2113-131 and BM2113-143 alleles described by MacHugh (1996) were observed with allelic frequencies of 0.488 and 0.064, respectively. TGLA53 genotype determination was doubtful due to three stutters per allele and the unbalanced size of alleles. To detect any possible SNP that could inhibit the binding of the primers for the amplification of this microsatellite locus, 122 bases upstream and 92 bases downstream of the flanking regions were sequenced. No SNPs were detected so finally TGLA53 was excluded from the study.

Table 2 *Statistical parameters of Spanish Salers sample based on 11 microsatellite loci*

	Na	HW	Ho	He	F_{IS}
BM1818	7	0.831	0.340	0.324	−0.049
BM1824	5	0.231	0.720	0.729	0.013
BM2113	9	0.322	0.730	0.710	−0.027
ETH10	7	0.784	0.744	0.714	−0.042
ETH225	5	0.117	0.772	0.770	−0.002
ETH3	8	0.230	0.576	0.583	0.013
INRA023	8	0.014	0.695	0.731	0.049
SPS115	6	0.013	0.201	0.211	0.046
TGLA122	9	0.517	0.687	0.696	0.013
TGLA126	5	0.099	0.588	0.590	0.003
TGLA227	12	0.000***	0.742	0.769	0.036
Average	7.36	0.000***	0.618	0.621	0.005

Na = number of alleles at the locus; HW = Hardy–Weinberg equilibrium probability (Bonferroni's correction $P < 0.0045$); Ho = observed heterozygosity; He = expected heterozygosity; F_{IS} = inbreeding coefficient.
***$P < 0.001$.

HW equilibrium was respected in all markers when Bonferroni's correction was applied, except for the locus TGLA227 (Table 2). The expected heterozygosity across all markers varied from 0.211 (SPS115) to 0.770 (ETH225), where the average expected heterozygosity was 0.621. TGLA227 showed deficit of heterozygotes but was not significant ($F_{IS} = 0.036$, $P > 0.05$), whereas BM1818, BM2113, ETH10 and ETH225 showed non-significant heterozygote excess. When all loci were combined, Salers inbreeding coefficient did not differ statistically from 0 ($F_{IS} = 0.005$), indicating not significant excess or deficit of heterozygotes ($P > 0.0045$). The absence of significant deviations from HW equilibrium shows that the Spanish Salers do not seem to be affected by the low number of founder individuals and its fast growing population.

Founder effect analysis

BOTTLENECK software was used to study a possible founder effect in Spanish Salers (Table 3). Sign test and Wilcoxon's sign rank test were performed under SMM. TPM of mutation

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Table 3 *Different tests for founder effect analysis in Salers cattle*

Test	TPM	SMM
Sign test		
Number of loci with He excess	2	2
Number of loci with He deficiency	9	9
P value	0.008	0.007
Wilcoxon's sign rank test		
P value (one-tailed test for He excess)	0.517	0.994

TPM = two-phase model; SMM = stepwise mutation model; He = heterozygosity.

was also applied as most microsatellite data sets fit the TPM better than other mutation models (Di Rienzo *et al*., 1994). Sign test showed heterozygosity excess in two loci under SMM ($P = 0.007$) and TPM ($P = 0.008$), although these results were not significant when Bonferroni's correction for multiple comparisons was applied ($P < 0.0045$). The one-tailed Wilcoxon's sign rank test revealed no significant deviation from mutation-drift equilibrium. The mode-shift indicator test was also utilized as a second method to detect potential founder effect. Graphical representation using allelic class and proportion of alleles showed a normal L-shaped distribution where alleles with low frequencies were the most abundant (Supplementary Figure S1). The performed tests verify that Spanish Salers individuals do not show founder effect although it has been recently introduced into the Iberian Peninsula. This is probably due to a satisfactory crossing procedure with the incorporation of new Salers reproducers to this population, as revealed by the observed inbreeding coefficient ($F_{IS} = 0.005$).

Genetic variation and relationship among breeds

Allele frequencies from European breeds were taken from Van de Goor *et al.* (2011) and used for the calculation of diversity parameters as described in the 'Material and methods' section. French Salers allele frequencies were not available for the same panel of microsatellite markers used in this study. However, diversity parameters published (observed heterozygosity, expected heterozygosity, number of allele per locus, F_{ST} and R_{ST}) from Amigues *et al.* (2011) were used to compare our Spanish population and French Salers. The mean number of alleles per locus for Spanish Salers was 7.36 (Table 1), considerably higher than the value for all breeds, being only lower than Holstein, Friesian and Limousin values (data not shown). The allelic richness was computed, as an estimate of the number of alleles per locus corrected by sample size, that allows a better comparison among breeds (Supplementary Table S1). This value was also higher in Spanish Salers (5.129) than the mean value calculated for all European breeds (4.985). In addition, other French breeds also showed higher allelic richness than this mean value.

African zebu alleles were found in French breeds as in our Spanish Salers sample. BM2113-131 allele, highly present in Spanish Salers (0.488), was also present in Limousin (0.222), Blonde d'Aquitaine (0.124) and Charolais (0.054). BM2113-143 allele was higher in French Blonde d'Aquitaine (0.167) than in Spanish Salers (0.064), whereas it was lower in Limousin (0.036). In contrast, these alleles were not found in other breeds, except Waldviertler breed that showed a high frequency (0.611) in BM2113-131. The results presented above could be of interest to support previous hypotheses (Cymbron *et al*., 2005) about the spread of agropastoralism across France.

Average expected heterozygosity varied in European breeds from 0.576 in Heck to 0.736 in Limousin cattle (Supplementary Table S2). Spanish Salers heterozygosity (0.621) was lower than the mean value for all breeds (0.661), Limousin (0.736) and Blonde d'Aquitaine (0.729) but it was close to Charolais (0.622). Value obtained for French Salers by Amigues *et al.* (2011) was 0.73, higher than Spanish Salers even though they analyzed a lower number of individuals.

Data of European cattle breeds from Van de Goor *et al.* (2011) and our Spanish Salers data were used to test genetic substructure. The global deficit of heterozygotes across all populations (F_{IT}) amounted to 10.9%. The estimated mean values for F_{ST} (0.106) were similar to the absolute values determined by R_{ST} (0.105) indicating that 10.5 % of the total genetic variation corresponds to differences among the breeds and 89.5% arose from differences among individuals (Supplementary Table S3). Differences in Reynolds genetic distances for each pair of breeds were mostly significant ($P < 0.001$) which means that the breeds can be considered genetically independent (Supplementary Table S4). Salers breed have shown to be genetically more similar to Blonde d'Aquitaine ($R_{ST} = 0.061$) than to other French breeds (Limousin, $R_{ST} = 0.084$; Charolais, $R_{ST} = 0.142$). Figure 1 shows the graphic representation of the NJ phylogenetic tree constructed with Reynolds distances. Calculated R^2 was 0.933, being close to 1.0 which means that the statistical significance represented in the tree was a good summary of the genetic relationships shown in the distance matrix as described by Kalinowski (2009). The NJ tree displayed a branch where Spanish Salers grouped with French breeds (Blonde d'Aquitaine and Limousin) and Waldviertler breed. Charolais was the only French breed that was not included in this branch as it showed to be closer to Belgian Blue and other Dutch breeds.

Clustering analysis using a Bayesian approach (STRUCTURE 2.3.1) was performed with an increasing number of inferred clusters ($K = 1$ to 21). Salers breed does not show a partitioning of genetic variability according to geographical origin. All Salers individuals showed a membership that was equally distributed among the Salers cluster, with the exception of few animals from a particular herd of this Spanish population. For all breeds, the potential structure is displayed in the Figure 2. Structure's posterior probabilities of *K*, as a log-likelihood given *K* clusters and the corresponding ΔK statistic (Evanno *et al*., 2005), showed maximal ΔK occurred at $K = 2$ (Supplementary Figure S2) and 95.9% of animals were attributed to one of the two clusters using a cutoff value of 80% ancestry for assignment. These clusters were inferred only on the basis of allele frequency differences. Furthermore, a second structure analysis was conducted to study the model clustering using a smaller sample for Friesian Holstein and

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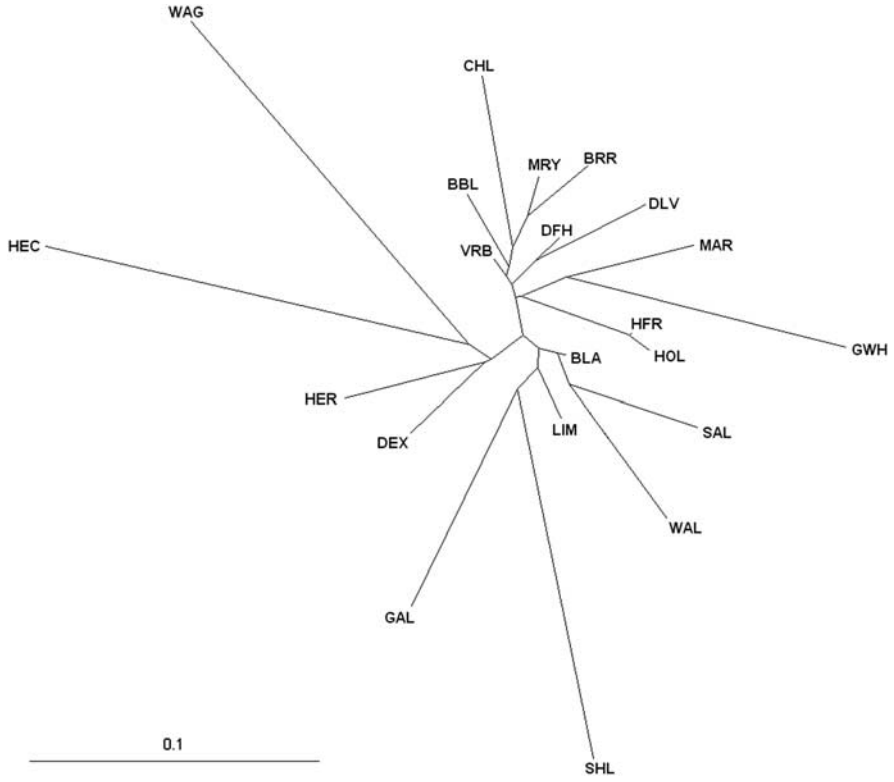

Figure 1 Neighbor-joining tree based on the Reynolds genetic distances between Salers and other breeds.

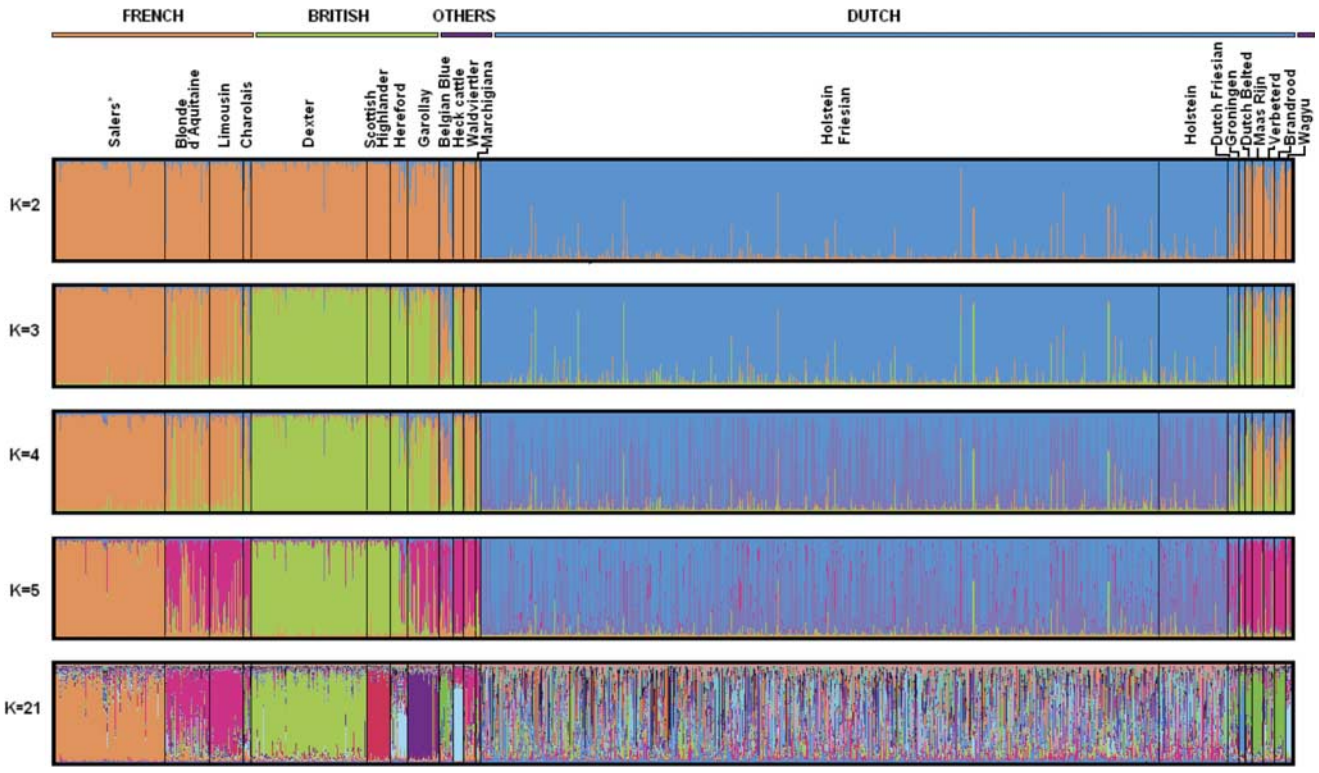

Figure 2 Population partitioning suggested by STRUCTURE based on the 11 microsatellites. Each individual animal is represented by a single vertical line divided into *K* colors, where *K* is the number of clusters assumed, and colored segment represents the individual's estimated proportion of membership to a particular cluster. Black lines separate individual populations whose names are indicated above the diagram.

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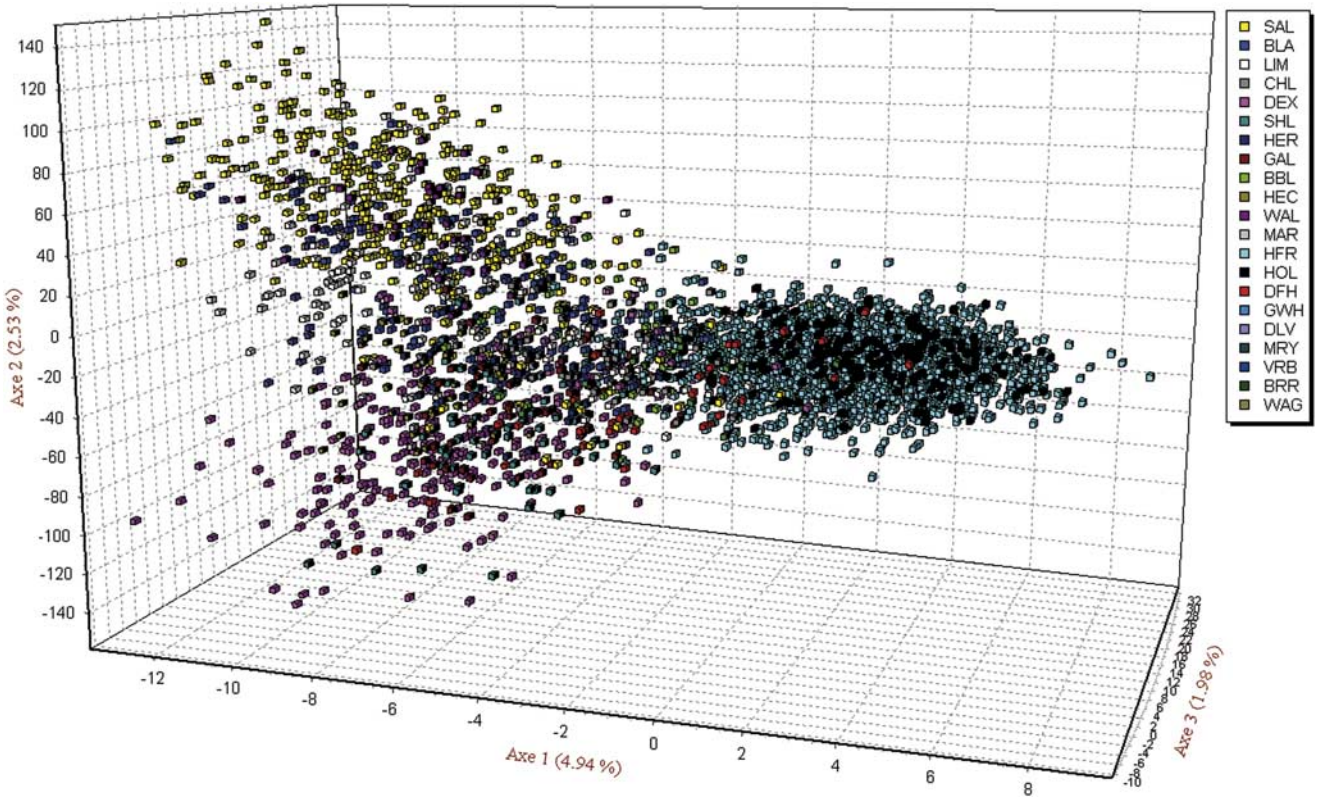

Figure 3 Plot of multivariate correspondence analysis calculated over all individuals for 21 populations.

Holstein breeds whose initial large samples could have disturbed the global analysis. In this case, ΔK was also observed at $K = 2$ and with the cutoff value of 80% ancestry for assignment, 89.6% of animals were attributed to one of the two clusters: 1130 individuals to a cluster where French breeds were grouped and 666 individuals to the other cluster. CA plot was performed including all animals (Figure 3). A total 9.45% of variance was accounted by the first three dimensions. Axis 1 separates French and British islands breeds (positive) from Holstein and Friesian (negative), while Axis 2 separates French breeds (positive) and British breeds (negative).

Discussion

The Salers breed has been widely introduced to many countries showing a high population increase due to its good productive characteristics and excellent adaptive capacity. This study is the first genetic characterization and the first founder effect analysis of Spanish Salers as a foreign breed in the Iberian Peninsula that establishes phylogenic relationships with European breeds. We studied microsatellite loci as they are highly polymorphic, although those markers have also shown some difficulties for allele size calling (Sanz *et al.*, 2014). Thus, new advances in high-throughput sequencing and excellent repeatability in SNP markers (Tsuchihashi and Dracopoli, 2002) have originated consistent alternatives for genetic characterization in cattle (Gautier *et al.*, 2010). Even so, this study has been accomplished using microsatellite markers as they are still the standard in many breeding programs and wide bibliography has allowed us comparison with other breeds.

Genetic characterization of Spanish Salers breed

The genotyping was performed by the analysis of 12 loci microsatellites, but the TGLA53 microsatellite locus did not render results. The region of attachment of primers was sequenced to study if some SNP in primer sequence target could have inhibited the PCR amplification. However, no SNPs were detected interfering the binding of the primers. So, an explanation based on the DNA sequence of the flanking regions was not obtained. The marker was excluded from analysis due to the low and uncertain efficiency of genotype determination as previously described by other authors (Putnova *et al.*, 2011; Sanz *et al.*, 2014).

We only found heterozygote deficit in TGLA227 marker. Heterozygote deficit might be explained by inbreeding due to a small number of reproducers, genetic drift and (or) population substructures as described by Maudet *et al.* (2002). Thus, HW equilibrium was studied by cattle herd. TGLA227 heterozygote deficit was only found in one herd. This herd could have maintained certain reproductive isolation that could explain the genetic pattern encountered, although it was not extended to the total Spanish Salers sample.

Despite the difficulties in comparing literature results, as they have been obtained with different marker sets, the wide literature on genetic variability of cattle breeds shows that heterozygosity estimates vary from 0.600 to 0.750

(Dalvit *et al*., 2008). In Spanish Salers, average heterozygosity was 0.621, moderate in comparison with the values reported for other European breeds. Spanish Salers value was higher than value showed by Cymbron *et al.* (2005) in French Salers (0.55) and consistent to the value reported by Cañon *et al*. (2001) (0.631). Amigues *et al*. (2011) showed higher value (0.67), although these studies were analyzed with other markers and in smaller French samples. This author showed an average number of alleles per locus of 5.6, while our study had higher value (7.36) but sample size should also be considered.

Several publications have reported the presence of zebu and African-type microsatellite alleles in Iberian cattle breeds (Beja-Pereira *et al*., 2003; Ginja *et al*., 2009). Our study confirms the presence of African zebu-diagnostic alleles in Spanish Salers, alleles also observed in southern French breeds from Van de Goor *et al.* (2011). These African-type alleles are consistent with an African heritage also shown in mitochondrial DNA studies. T1 haplogroup, almost fixed in African cattle, has also been found in southern European breeds (Portugal, Spain, Greece and Italy) (Cymbron *et al*., 1999; Beja-Pereira *et al*., 2006; Lopez-Oceja *et al*., 2015) and in French Limousin (Achilli *et al*., 2009). This could suggest that North African cattle may have left a genetic signature in southern Europe based on the hypothesis of Neolithic dispersal through the Mediterranean route (Zilhao, 1993) and historical migrations (Cymbron *et al*., 1999). In agreement with those hypotheses, the presence of zebu BM2113-143 allele in Spanish Salers and French breeds as well as the high frequency of zebu BM2113-131 allele could be explained by the same zebu gene flow from the Mediterranean area. In fact, SNP data is in agreement with the postulated migration route by which the Neolithic culture expanded toward France as southwestern French breeds appear closely related to the Mediterranean colonization route (Gautier *et al*., 2010). In addition, NJ dendrogram of Figure 1 shows Reynolds genetic distances from Salers, Blonde d'Aquitaine and Limousin breeds where they group together probably due to their high frequency in African zebu allele BM2113-131.

Founder effect analysis

Salers breed has been recently introduced to Spain in 1985. The number of individuals coming from France was very low, so a potential genetic founder effect cannot be discarded. As showed in Table 3, the founder effect was investigated using the SMM as it assumes that a mutation results in a change in one repeat unit either by insertion or deletion. TPM was also considered because it allows mostly one-step mutations, but also a small percentage (5% to 10%) of multi-step changes. Sign test that suffers from low statistical power (Piry *et al*., 1999) revealed significant deviation under SMM and TPM models (uncorrected). However, after Bonferroni's correction ($P < 0.0045$), none of the models showed significant deviation. Using the Wilcoxon's rank test, a non-parametric test with relatively high power, the probability values were not significant under both models, thus rejects in both cases a founder effect. The mode-shift indicator test was also utilized as an additional method to detect a potential founder effect. This test discriminates populations with bottleneck or founder effect from stable populations as the populations without founder effect being near mutation-drift equilibrium, are expected to have a large proportion of alleles with low frequency. Normal L-shaped graphical representation where most abundant alleles had low frequencies (0.01 to 0.1) was observed, suggesting that studied population is under mutation-drift equilibrium (Supplementary Figure S1).

A reduction of gene diversity (He, or HW heterozygosity) (Böhme *et al*., 2007) could be expected owing to a founder effect, and also a reduction on the allele numbers (Na) (Piry *et al*., 1999). Gene diversity (He) observed in Spanish Salers (0.621) was slightly lower than French Salers (0.67) from Amigues *et al.* (2011), but allele number per locus was higher for Spanish Salers (7.36) than for French population (5.6). Therefore, the gene diversity results did not support a founder effect according to Spanish Salers. This is reinforced by the fit to HW equilibrium observed in Spanish Salers, except for one particular herd ($P = 0.0011$).

Although Salers has been recently introduced to Spain, constant incorporation of new reproducers from France could have increased the effective population. Furthermore, this breeding management could have avoided negative effects from the reduced founder population and a possible inbreeding. Thus, high genetic diversity in Spanish Salers highlights the resources of this population, which looks toward future breeding and selection programs.

Genetic variation and relationship among breeds

High heterozygosity was observed in French breeds (Limousin 0.736; Blonde d'Aquitaine 0.729 and Charolais 0.622) compared with Salers, whose heterozygosity estimate was reasonable compared with the values reported in American Salers by MacNeil *et al.* (2007) and much higher than observed by Cymbron *et al.* (2005) in French Salers.

Despite long population histories in their native countries and unique morphological traits, lack of controlled breeding in the second part of the 20th century resulted in some dispersion of related breeds. However, our results revealed that Salers appear to be more related to Blonde d'Aquitaine as previously described in biochemical polymorphisms (Grosclaude *et al*., 1990). Interestingly, Amigues *et al.* (2011) showed comparable genetic differentiation in French Salers and Blonde d'Aquitaine ($R_{ST} = 0.07$) and French Salers and Limousin ($R_{ST} = 0.07$) based on microsatellite data. Deficit of heterozygotes was also similar to Amigues *et al.* (2011) ($F_{IT} = 0.091$), when we computed it only across same breeds (Spanish Salers, Blonde d'Aquitaine, Limousin and Holstein) ($F_{IT} = 0.095$). Therefore, previous genetic differentiation and deficit of heterozygotes do not seem to be very different from our results. However, Charolais was unpredictably separated from this French group (Figure 1), but such results are not entirely unanticipated. Wiener *et al.* (2004) found relationship between breeds that they do not primarily cluster according to geographical origin and Gautier *et al.* (2010) displayed that Charolais branched with a less reliable node

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with breeds from southwestern France (Salers and Blonde d'Aquitaine). Scottish breeds (Highland and Galloway) do not show strong affinities with other breeds from the British Isles (Dexter and Hereford). In Hereford, it may reflect historical genetic input from northern Europe, which is known to have occurred before the establishment of the Hereford herd in 1846 (Heath Agnew, 1983). The Wagyu branch is in accordance with expected tracing the breed's origin to cattle from Japan. Meanwhile, Heck cattle is also shown apart from other European breeds as it is known that Heck cattle was the result of the first attempt to breed back domestic cattle to the ancestral aurochs (*Bos primigenius*).

A Bayesian MCMC approach, represented in Figure 2, was used to examine the distinctiveness of Salers and other breeds. STRUCTURE software genetic clusters and Evanno method showed a maximal ΔK at $K = 2$, indicating 2 as the most likely number of ancestral populations for the 21 breeds analyzed (Supplementary Figure S2). A clear distinction was identified between French breeds and British Isles breeds at $K = 3$, corresponding to their different geographical distributions. At $K = 3$, French breeds and Waldviertler breed were grouped together probably due to the African alleles described previously. In fact, a clear agreement of STRUCTURE clustering with the genetic distance analysis and consistent with major clusters of the NJ distance phenogram was observed in Figure 1. CA plot (Figure 3) was similarly in agreement with Bayesian structure method when $K = 3$ was observed (Figure 2). French breeds appeared to cluster separately from British Isle breeds, whereas other Dutch breeds were dispersed on the center of the plot but close to a big cluster of Holstein Friesian. Populations were mostly separated in correspondence to their geographical origin, although some exceptions were observed. For instance, similar pattern from structure analysis was shown in Waldviertler breed that was closer to Salers and French breeds. Structure from Amigues *et al.* (2011) differentiated Salers, Blonde d'Aquitaine and Limousin at $K = 4$. In contrast, our results showed that Blonde d'Aquitaine, Limousin and Charolais seems to be more related, as Charolais was only separated at $K = 10$, whereas Blonde d'Aquitaine and Limousin still clustered together at $K = 21$. At this level, British breeds are in different clusters showing high differentiation probably caused by their island isolation. Finally, dairy Friesian Holstein, Holstein and Dutch Friesian were assembled in the same cluster, whereas other Dutch breeds group together and Japanese Wagyu breed was singularly isolated. The large amount of Friesian Holstein and Holstein individuals in the present study could have disturbed clustering, therefore the sample size of these two breeds was reduced to $n = 100$, respectively (average population size for all breeds). In this case, Evanno method also showed same ancestral K at 2.

In conclusion, the current study is the first detailed analysis of the genetic diversity of Salers breed in the Iberian Peninsula. An interesting finding is the high frequencies of African alleles not previously reported in French Salers. This study reinforces the understanding of the genetic characterization of Salers and its relationships with European breeds. Finally, we found that the heterozygosity of Spanish Salers reveals the absence of a founder effect in the expansion of the breed to Spain indicating that economically important traits are probably preserved.

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Supplementary material

For supplementary material/s referred to in this article, please visit http://dx.doi.org/10.1017/S1751731116001063

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Supplementary Table S1. *Allelic Richness per locus and population based on minimum sample size (17 diploid individuals)*

	SAL	BLA	LIM	CHL	DEX	SHL	HER	GAL	BBL	HEC	WAL	MAR	HFR	HOL	DFH	GWH	DLV	MRY	VRB	BRR	WAG	Averag
BM1818	4.412	5.567	5.255	5.401	5.718	4.961	3.799	2.773	4.421	2.949	5.083	5.000	4.046	4.378	5.317	3.979	4.995	3.986	4.353	3.404	2.850	4.412
BM1824	4.671	3.998	3.692	3.973	3.989	2.788	4.002	3.999	3.965	3.313	4.760	2.000	4.697	4.709	3.961	5.546	4.000	3.805	3.649	4.459	3.850	3.992
BM2113	6.628	7.070	6.400	6.785	4.837	4.818	6.860	5.354	5.686	3.435	5.537	6.000	5.385	4.919	5.973	3.995	4.995	5.933	6.401	4.935	4.996	5.569
ETH10	5.139	4.464	5.834	3.496	5.130	3.000	3.965	3.750	5.341	3.000	2.755	4.000	6.000	5.843	6.048	4.974	5.918	4.303	3.787	4.321	4.550	4.553
ETH225	4.940	4.938	4.548	3.850	4.267	4.961	5.713	5.756	5.244	4.483	4.847	5.000	5.079	5.587	5.451	3.974	3.999	4.807	5.673	4.757	3.850	4.844
ETH3	4.410	4.499	5.308	5.772	5.641	3.921	2.475	3.785	7.342	3.997	4.315	4.000	4.864	4.779	6.152	4.687	2.979	5.406	5.704	4.527	4.831	4.733
INRA023	6.662	6.480	6.283	6.346	6.993	3.758	4.409	6.936	7.023	3.948	5.111	5.000	5.438	5.180	7.241	4.953	6.331	4.655	7.277	5.429	3.700	5.674
SPS115	3.233	4.807	5.118	4.455	6.155	3.661	5.586	5.338	3.114	1.999	4.604	4.000	4.472	4.781	4.019	3.547	1.979	2.999	4.430	3.000	5.000	4.109
TGLA122	5.556	7.137	7.717	7.122	4.877	4.472	6.244	5.181	6.023	5.818	4.142	4.000	7.851	7.386	7.436	4.396	6.627	6.426	8.562	5.181	4.700	6.041
TGLA126	3.331	4.089	4.986	2.997	3.559	2.989	4.271	2.931	4.247	4.509	3.372	5.000	4.478	4.443	4.374	3.999	4.918	4.411	5.080	5.352	3.000	4.111
TGLA227	7.437	7.426	7.509	8.271	6.230	4.890	6.632	7.393	6.626	5.622	7.487	6.000	8.017	7.861	7.041	4.989	6.390	7.087	6.673	7.198	5.960	6.797
Average	5.129	5.498	5.695	5.315	5.218	4.020	4.905	4.836	5.367	3.916	4.728	4.545	5.484	5.442	5.728	4.458	4.830	4.893	5.599	4.778	4.299	4.985

Supplementary Table S2. Expected *heterozygosities for the 11 loci in Salers breed and the comparison breeds*

Locus	SAL	BLA	LIM	CHL	DEX	SHL	HER	GAL	BBL	HEC	WAL	MAR	HFR	HOL	DFH	GWH	DLV	MRY	VRB	BRR	WAG	Averag
BM1818	0.324	0.640	0.662	0.694	0.767	0.732	0.624	0.481	0.490	0.535	0.555	0.722	0.582	0.617	0.715	0.588	0.789	0.655	0.653	0.584	0.535	0.616
BM1824	0.729	0.728	0.657	0.627	0.716	0.288	0.653	0.739	0.677	0.257	0.729	0.515	0.757	0.723	0.694	0.536	0.744	0.687	0.674	0.645	0.619	0.638
BM2113	0.710	0.851	0.806	0.807	0.713	0.751	0.803	0.507	0.781	0.607	0.592	0.709	0.758	0.724	0.810	0.671	0.783	0.815	0.845	0.746	0.704	0.738
ETH10	0.714	0.733	0.775	0.232	0.743	0.620	0.696	0.430	0.664	0.669	0.303	0.544	0.633	0.684	0.777	0.660	0.803	0.656	0.571	0.666	0.385	0.617
ETH225	0.770	0.765	0.706	0.618	0.710	0.752	0.786	0.758	0.749	0.627	0.633	0.702	0.717	0.715	0.683	0.600	0.673	0.603	0.754	0.539	0.596	0.688
ETH3	0.583	0.695	0.672	0.720	0.697	0.601	0.519	0.371	0.828	0.669	0.619	0.558	0.635	0.658	0.677	0.704	0.430	0.776	0.704	0.400	0.637	0.626
INRA023	0.731	0.777	0.794	0.734	0.782	0.575	0.573	0.787	0.818	0.681	0.714	0.774	0.763	0.746	0.839	0.536	0.761	0.612	0.810	0.667	0.458	0.711
SPS115	0.211	0.523	0.716	0.490	0.778	0.627	0.780	0.740	0.319	0.245	0.668	0.469	0.625	0.613	0.370	0.198	0.120	0.545	0.507	0.604	0.776	0.520
TGLA122	0.696	0.832	0.819	0.693	0.421	0.583	0.708	0.476	0.716	0.804	0.656	0.561	0.804	0.831	0.797	0.580	0.819	0.784	0.819	0.703	0.683	0.704
TGLA126	0.590	0.656	0.673	0.413	0.660	0.559	0.727	0.548	0.620	0.550	0.604	0.793	0.645	0.616	0.526	0.593	0.723	0.711	0.685	0.778	0.524	0.628
TGLA227	0.769	0.817	0.818	0.818	0.763	0.751	0.758	0.844	0.793	0.688	0.828	0.742	0.855	0.823	0.840	0.707	0.704	0.818	0.759	0.831	0.701	0.782
Average	0.621	0.729	0.736	0.622	0.705	0.622	0.693	0.607	0.678	0.576	0.627	0.644	0.707	0.705	0.703	0.579	0.668	0.697	0.707	0.651	0.602	0.661

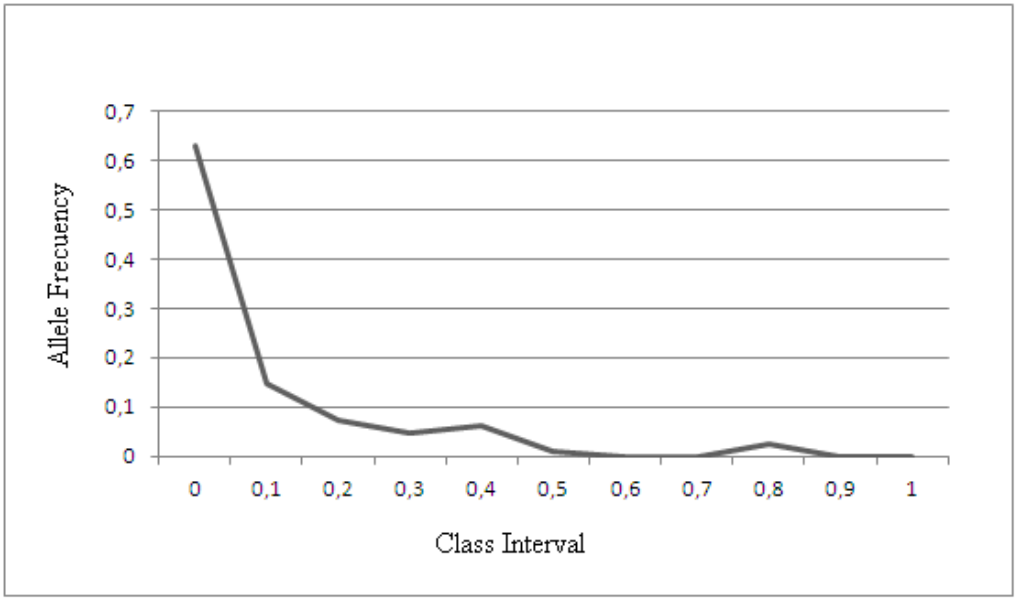
Supplementary Table S3. *Wright F-Statistics (FIT. FST. FIS) and breed differentiation (RST) among 21 breeds.*

	FIT	FST	FIS	RST
BM1818	0.100	0.097	0.003	0.092
BM1824	0.077	0.071	0.006	0.037
BM2113	0.147	0.138	0.011	0.126
ETH10	0.120	0.121	-0.002	0.057
ETH225	0.066	0.064	0.002	0.070
ETH3	0.077	0.076	0.001	0.096
INRA023	0.099	0.107	-0.009	0.103
SPS115	0.108	0.116	-0.009	0.177
TGLA122	0.144	0.134	0.011	0.111
TGLA126	0.170	0.152	0.022	0.065
TGLA227	0.090	0.084	0.006	0.112
Mean	0.109	0.106	0.004	0.105

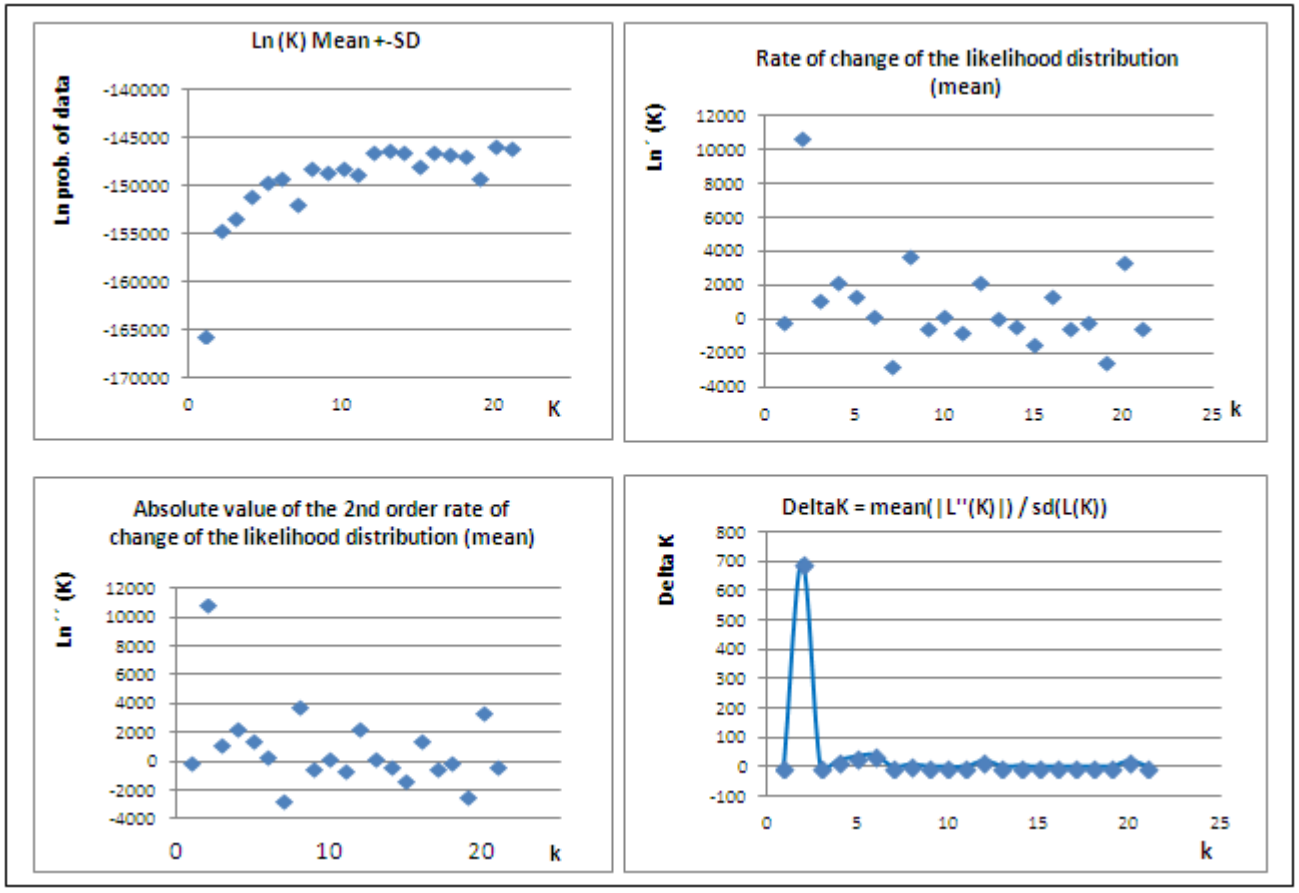
Supplementary Table S4. *Pairwise estimates of genetic differentiation and genetic distances among all the breeds. The FST estimates are above the diagonal and the Reynolds genetic distance is below the diagonal. All estimates of FST were found to be significant (P <0.01).*

	SAL	BLA	LIM	CHL	DEX	SHL	HER	GAL	BBL	HEC	WAL	MAR	HFR	HOL	DFH	GWH	DLV	MRY	VRB	BRR	WAG
SAL	*	0.060	0.081	0.133	0.128	0.203	0.134	0.163	0.081	0.224	0.098	0.103	0.125	0.139	0.119	0.183	0.134	0.111	0.083	0.137	0.251
BLA	0.061	*	0.025	0.084	0.069	0.154	0.075	0.124	0.051	0.161	0.067	0.076	0.073	0.077	0.055	0.136	0.068	0.055	0.049	0.075	0.152
LIM	0.085	0.026	*	0.106	0.088	0.134	0.092	0.122	0.074	0.202	0.079	0.076	0.098	0.103	0.070	0.159	0.079	0.069	0.066	0.089	0.156
CHL	0.142	0.088	0.113	*	0.117	0.215	0.153	0.170	0.089	0.200	0.098	0.148	0.127	0.128	0.074	0.194	0.113	0.098	0.075	0.078	0.216
DEX	0.136	0.071	0.092	0.125	*	0.150	0.082	0.152	0.101	0.172	0.133	0.096	0.101	0.112	0.088	0.152	0.097	0.100	0.080	0.100	0.182
SHL	0.227	0.168	0.144	0.242	0.162	*	0.171	0.193	0.204	0.289	0.204	0.200	0.169	0.181	0.175	0.253	0.198	0.194	0.175	0.214	0.242
HER	0.144	0.078	0.096	0.166	0.086	0.188	*	0.159	0.104	0.188	0.159	0.113	0.087	0.104	0.107	0.154	0.123	0.117	0.086	0.126	0.193
GAL	0.177	0.132	0.130	0.187	0.165	0.214	0.173	*	0.157	0.278	0.161	0.125	0.117	0.139	0.132	0.193	0.148	0.154	0.107	0.136	0.258
BBL	0.085	0.052	0.077	0.093	0.107	0.228	0.110	0.171	*	0.199	0.117	0.095	0.078	0.086	0.063	0.134	0.089	0.052	0.041	0.083	0.196
HEC	0.254	0.175	0.225	0.223	0.189	0.341	0.209	0.325	0.223	*	0.262	0.231	0.212	0.217	0.161	0.243	0.200	0.206	0.173	0.227	0.257
WAL	0.104	0.069	0.082	0.103	0.142	0.229	0.173	0.176	0.124	0.304	*	0.153	0.131	0.135	0.122	0.227	0.146	0.114	0.118	0.103	0.218
MAR	0.109	0.079	0.079	0.160	0.101	0.223	0.120	0.133	0.100	0.263	0.166	*	0.106	0.122	0.097	0.134	0.119	0.115	0.081	0.122	0.245
HFR	0.133	0.076	0.103	0.136	0.107	0.185	0.091	0.124	0.081	0.238	0.140	0.112	*	0.009	0.069	0.127	0.094	0.081	0.054	0.097	0.172
HOL	0.150	0.081	0.108	0.137	0.119	0.200	0.110	0.150	0.090	0.244	0.145	0.130	0.009	*	0.066	0.144	0.108	0.085	0.061	0.102	0.169
DFH	0.127	0.057	0.073	0.076	0.092	0.192	0.113	0.141	0.065	0.176	0.130	0.102	0.071	0.068	*	0.130	0.052	0.062	0.029	0.068	0.169
GWH	0.202	0.146	0.174	0.215	0.165	0.292	0.168	0.215	0.144	0.278	0.257	0.144	0.136	0.156	0.139	*	0.164	0.138	0.104	0.158	0.305
DLV	0.144	0.071	0.082	0.120	0.102	0.221	0.132	0.160	0.093	0.223	0.158	0.126	0.099	0.115	0.053	0.180	*	0.089	0.068	0.080	0.212
MRY	0.117	0.056	0.072	0.103	0.105	0.216	0.124	0.167	0.054	0.231	0.121	0.122	0.084	0.089	0.064	0.148	0.094	*	0.026	0.040	0.191
VRB	0.087	0.050	0.068	0.078	0.083	0.192	0.090	0.114	0.042	0.190	0.125	0.084	0.055	0.063	0.030	0.110	0.071	0.027	*	0.055	0.194
BRR	0.147	0.078	0.094	0.081	0.105	0.241	0.134	0.146	0.087	0.258	0.109	0.130	0.102	0.107	0.070	0.172	0.084	0.041	0.057	*	0.219
WAG	0.288	0.165	0.170	0.244	0.201	0.277	0.215	0.298	0.218	0.297	0.246	0.282	0.189	0.186	0.186	0.364	0.238	0.212	0.215	0.247	*

Supplementary Figure S1. *L-shaped mode shift graph showing the absence of founder effect in Spanish Salers cattle.*



Supplementary Figure S2. *Evanno method representation to obtain the most likelihood cluster from Structure analysis.*



APPENDIX III.

PUBLICATION III.

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Genetic Characterization of the Local Pirenaica Cattle for Parentage and Traceability Purposes

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Simple Summary: Domestic livestock diversity is an important component of global biodiversity and molecular data have become essential for the characterization of genetic diversity in cattle. The aim of this study was to assess the effectiveness of a 30-short tandem repeat (STR) panel and reveal the genetic structure of a local Pirenaica breed compared with other breeds (Terreña, Blonde d'Aquitaine, Limousin, Salers and Holstein-Friesian) typically raised in the same geographic Basque region. The proposed STR panel could be used as an appropriate genetic tool to trace Pirenaica animals and their Protected Geographic Indication (PGI) products.

Abstract: Pirenaica is the most important autochthonous cattle breed within the Protected Geographic Indication (PGI) beef quality label in the Basque region, in northern Spain. The short tandem repeats (STRs) are powerful markers to elucidate forensic cases and traceability across the agri-food sector. The main objective of the present work was to study the phylogenetic relationships of Pirenaica cattle and other breeds typically raised in the region and provide the minimum number of STR markers for parentage and traceability purposes. The 30-STR panel recommended by the International Society of Animal Genetics-Food and Agriculture Organization of the United Nations (ISAG-FAO) was compared against other commercial STR panels. The 30-STR panel showed a combined matching probability of 1.89×10^{-25} and a power of exclusion for duos of 0.99998. However, commercial STR panels showed a limited efficiency for a reliable parentage analysis in Pirenaica, and at least a 21-STR panel is needed to reach a power of exclusion of 0.9999. Machine-learning analysis also demonstrated a 95% accuracy in assignments selecting the markers with the highest F_{ST} in Pirenaica individuals. Overall, the present study shows the genetic characterization of Pirenaica and its phylogeny compared with other breeds typically raised in the Basque region. Finally, a 21-STR panel with the highest F_{ST} markers is proposed for a confident parentage analysis and high traceability.

Keywords: structure; identity; assignment test; microsatellite; multiplex PCR; Salers; Holstein-Friesian; Terreña; Blonde d´Aquitaine; Limousin

1. Introduction

Pirenaica are the most important beef cattle raised in the Basque region, northern Spain, and their meat is included, together with other breeds, within the local Protected Geographic Indication (PGI) label [1]. The regulation of the PGI quality label establishes the production system and defines

health and welfare requirements. This beef is highly appreciated by the local consumer, and has been registered as an "100% autochthonous breed" by the Spanish Ministry of Agriculture under the National Regulation (R.D 2129/2008, December 26th) on conservation, improvement and promotion of animal breeds [2]. Tool-supported traceability is of high importance to avoid food fraud and maintain high standards across the agri-food industry. In this sense, DNA-based traceability constitutes a powerful tool for parentage detection, and individual or species identification in the food chain [3,4]. For instance, microsatellite, or short tandem repeat (STR) loci, have become necessary tools for pedigree recording and inbreeding control and are essential for conservation and selection programs [5]. STR loci are also interesting for individual animal identification purposes through the meat chain promoting food safety and traceability [6,7].

In this sense, several STR panels have been proposed for parentage verifications in the breeding industry and cattle related forensic cases. However, depending on the studied breed, a selection of an appropriate STR panel with a minimum number of markers allowing a kinship exclusion probability over 99.99 is required. In this sense, initially, a panel of 9-STR was recommended suggesting the increase of markers in parentage testing [8], and two markers were included in a commercial 11-STR panel (StockMarks®, Applied Biosystems, Foster City, CA, USA). Later, a 12-STR core panel was proposed [9] and commercialized (Bovine Genotype™ Panel 1.2, Thermo Scientific, Waltham, MA, USA). Finally, six additional markers were intended (Bovine Genotype™ Panel 2.2, Thermo Scientific, USA) to be used as a complement to Panel 1.2 when more STR loci are required. For cattle genetic resources management studies, on the other hand, FAO proposed a 30-STR panel [10]. The 12-STR core panel has been widely used in parentage and identity testing, but after the poor discrimination power observed among several European breeds it was suggested to increase the amount of genotyped STRs for forensic cases [11]. In the Pirenaica breed, a 10-STR panel was studied for traceability purposes [12] and other few and/or not core STRs were reported for genetic diversity [13,14]. In other breeds included in the Basque PGI label, such as autochthonous Terreña, or imported Blonde d´Aquitaine, Limousin and Salers, genetic diversity studies have been reported [15–17]. However, most of previous studies were performed before the recommendation by the FAO for using a 30-STR panel, and therefore, limitations of markers make for a difficult the comparison with data from later studies [10]. Therefore, the objectives of the present study were (1) to study the genetic diversity and phylogeny of Pirenaica cattle; (2) to assess the effectiveness of the 30-STR panel, from the International Society of Animal Genetics-Food and Agriculture Organization of the United Nations (ISAG-FAO) Advisory group on Animal Genetic Diversity, for parentage and individual identification compared to other commercial STR panels recognized as the minimum standard for identity and kinship testing; and (3) to assess the traceability through genetic assignments comparing several STR panels in a single predictive model and select the most discriminative markers to determine the population of origin of an unknown individual in Pirenaica and other breeds raised in the Basque region (Terreña, Blonde d'Aquitaine, Limousin, Salers and Holstein-Friesian).

2. Materials and Methods

2.1. Sample Collection and DNA Extraction

Muscle samples from Pirenaica beef cattle from several farms located in the Basque Country (northern Spain) were collected (n = 114) according to the Bovine Identification Document. Neither parentage nor maternal half-sibs were observed, and paternal half-sibs were maintained at low frequencies (0.009). Furthermore, muscle samples from Salers (n = 13) and Holstein-Friesian cattle (n = 21) were collected for assignment accuracy purposes [18]. Salers and Holstein-Friesian (as cull dairy cows) are both integrated in the Basque beef supply chain. Pirenaica, Salers and Holstein-Friesian were purebred and, in all cases, neck (*Sternomandibularis)* muscle samples were collected at 24 h *postmortem* in a local commercial abattoir (Harakai Urkaiko S. Coop., Zestoa, Gipuzkoa). DNA was extracted from 20 mg of muscle tissue using a salting-out method. The DNA pellet was re-hydrated with 200 µL of

H_2O and aliquots were stored at −20 °C. DNA samples were quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and diluted to 50 ng/µL.

2.2. Sample Genotyping

The PCR amplification of the recommended 30 microsatellites (BM1824, BM2113, ETH03, ETH010, ETH225, INRA023, SPS115, TGLA53, TGLA122, TGLA126, TGLA227, BM1818, CSSM66, CSRM60, ILSTS006, HAUT27, HEL01, INRA005, INRA037, INRA063, ETH152, HEL09, ETH185, HEL05, HEL13, INRA032, MM12, HAUT024, ILST005 and INRA035) was performed by multiplex PCR reaction [10]. Capillary electrophoresis was performed on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using internal size standard GS LIZ500 (Applied Biosystems). GeneMapper v4.0 was used for fragment analysis.

2.3. Statistical Analysis

In order to study the genetic variation, Genepop 4.2 software [19] was used to test for deviations from Hardy–Weinberg equilibrium (HW) using the test reported by Guo and Thompson [20] and a Markov chain (dememorization 5000, batches 100, iterations per batch 1000). Bonferroni's procedure was applied to correct the level of significance of multiple tests. The cervus 3.0.3 software [21] was used to calculate the number of alleles per locus (k), observed heterozygosity (Ho) and expected heterozygosity (He).

Genetic relationships of Pirenaica were analyzed against Terreña, Salers and Holstein-Friesian STR data from our previous studies [15,16]. Reynolds genetic distance measures were computed by Arlequin 3.5 [22]. A factorial components analysis (FCA) was used to represent a three-dimensional plot [23], and a Neighbor-joining (NJ) phylogenetic tree based on individuals was also constructed [24].

For individual identification and traceability, matching probability (MP) was computed as the probability to have a match between two individuals sharing an identical genotype profile and chosen at random [25] and using Powerstats 1.2 (Promega, Madison, WI, USA). The combined matching probability (CMP) was computed with the formula: CMP = $(MP_1)(MP_2) \ldots (MP_k)$ which is the overall MP including k number of loci. For parentage purposes, power of exclusion for each locus (PE) was calculated in the absence of genetic information from one parent (PE-1P) and power of exclusion when genetic information of both parents was available (PE-2P), power of exclusion for identity of two siblings (PE-SI) was also computed. Finally, combined power of exclusion (CPE) with the formula: CPE = $1 - (1 - PE_1)(1 - PE_2) \ldots (1 - PE_k)$ which is the overall PE including k number of loci was calculated [26]. CPE was calculated for PE-1P (CPE_1), PE-2P (CPE_2) and PE-SI (CPE_{SI}) using several commercial marker panels and compared with the improved discrimination power of the 30-STR panel.

Assignment tests were performed including several breeds typically raised in the Basque region. On one hand, minority PGI breeds such as native Terreña [15], Blonde d´Aquitaine and Limousin [11] were included using published STR data. On the other hand, Salers and Holstein-Friesian breeds, genotyped in this study using the 30-STR panel, were included. First of all, the assignment of individuals to their breeds was tested using the frequency-based [27] and the Bayesian-based [28] methods implemented in geneclass 2 software [29]. The Bayesian method was computed by simulating 1000 genotypes (using allele frequencies) and a fixed threshold of 0.001. Thus, an individual was considered as correctly assigned to a population when it was excluded from all of the non-origin populations ($p \leq 0.001$), but not from the true population of origin. Secondly, the existence of distinct genetic populations and assignment was tested with a Bayesian model based method in structure 2.3.4 software [30] under an admixture model for clusters (K) and using 10 Markov Chain Monte Carlo simulations consisting of 1×10^5 iterations after a burn-in of 5×10^5 iterations. The optimal value of K was selected following the clustering mode described by Kopelman et al. [31] and the approach ΔK of Evanno et al. [32]. Thereafter, a supervised machine-learning approach was used to estimate the mean and variance of assignment accuracy by a Monte-Carlo resampling (100 iterations) cross-validation procedure using r software and AssignPOP package [33]. Analyses were adjusted by the proportion of

individuals and by the STRs with the highest F_{ST} to estimate the minimal number of markers for an accurate assignment. This approach creates randomly selected, independent training and test data panels which avoids introducing high-grading bias [34], while the proportion of individuals from each source population randomly allocated to the baseline data panel was adjusted to avoid biases associated with unbalanced population sizes [35].

3. Results and Discussion

3.1. Genetic Variations and Genetic Relationships

A total of 30 STR loci were analyzed in 114 Pirenaica individuals and HW equilibrium was reached in all markers when Bonferroni's correction was applied, except for the locus INRA037 (Table 1). The He across all markers varied from 0.372 (ILST005) to 0.865 (TGLA227), where the average He was 0.680. All the loci were polymorphic and 211 alleles were detected. The number of alleles per locus ranged between 2 (ILST005) and 12 (CSSM66) with an average of 7.03 ± 2.51.

Table 1. Statistical parameters for genetic characteristics of Pirenaica breed using the panel of 30 short tandem repeat markers by ISAG-FAO [10].

Marker	k	Ho	He	PIC	MP	PE-1P	PE-2P	PE-SI	HW
BM1824	4	0.711	0.745	0.694	0.114	0.319	0.668	0.592	NS
BM2113	8	0.816	0.824	0.797	0.062	0.469	0.821	0.646	NS
ETH03	8	0.588	0.621	0.582	0.181	0.224	0.597	0.513	NS
ETH010	6	0.746	0.710	0.658	0.160	0.295	0.649	0.570	NS
ETH225	6	0.684	0.639	0.604	0.161	0.241	0.623	0.527	NS
INRA023	7	0.737	0.769	0.729	0.088	0.373	0.736	0.610	NS
SPS115	6	0.596	0.581	0.522	0.235	0.182	0.503	0.481	NS
TGLA53	11	0.754	0.820	0.791	0.063	0.462	0.815	0.643	NS
TGLA122	11	0.746	0.768	0.734	0.084	0.387	0.758	0.611	NS
TGLA126	5	0.658	0.608	0.556	0.201	0.203	0.545	0.502	NS
TGLA227	11	0.860	0.865	0.845	0.039	0.559	0.882	0.672	NS
BM1818	7	0.728	0.783	0.750	0.080	0.401	0.770	0.620	NS
CSSM66	12	0.732	0.777	0.755	0.078	0.422	0.808	0.619	NS
CSRM60	7	0.652	0.632	0.602	0.163	0.240	0.633	0.523	NS
ILSTS006	7	0.719	0.806	0.774	0.070	0.432	0.789	0.634	NS
HAUT27	7	0.693	0.745	0.702	0.111	0.342	0.706	0.594	NS
MM12	11	0.842	0.804	0.777	0.086	0.445	0.810	0.634	NS
HEL09	7	0.754	0.798	0.764	0.075	0.417	0.776	0.629	NS
INRA032	8	0.693	0.747	0.710	0.099	0.351	0.725	0.597	NS
ETH152	6	0.788	0.746	0.701	0.124	0.336	0.696	0.594	NS
HAUT024	8	0.772	0.737	0.691	0.123	0.327	0.687	0.589	NS
INRA037	9	0.268	0.663	0.601	0.231	0.250	0.588	0.536	***
INRA005	4	0.604	0.642	0.564	0.198	0.206	0.500	0.518	NS
ETH185	8	0.536	0.596	0.554	0.222	0.202	0.563	0.496	NS
HEL05	8	0.561	0.567	0.535	0.217	0.185	0.555	0.477	NS
INRA063	4	0.563	0.576	0.506	0.243	0.172	0.469	0.474	NS
HEL01	5	0.554	0.558	0.505	0.254	0.165	0.486	0.466	NS
HEL13	5	0.482	0.469	0.421	0.352	0.113	0.400	0.401	NS
INRA035	3	0.298	0.427	0.384	0.397	0.091	0.357	0.370	NS
ILST005	2	0.351	0.372	0.302	0.463	0.069	0.237	0.319	NS

k: number of alleles per locus; Ho: observed homozygosity; He: expected heterozygosity; PIC: polymorphic information content; MP: matching probability; PE-1P: power of exclusion for 1 known parent; PE-2P: power of exclusion for 2 known parents; PE-SI: power of exclusion for sibling; HW: Hardy–Weinberg equilibrium; NS: non-significant; ***, $p < 0.001$.

The origin of Iberian breeds occurred through arrival of cattle from the *Bos taurus* (Taurine) lineage. However, MacHugh [36] observed African zebu (*Bos indicus*) diagnostic alleles in European and African taurine (*Bos taurus*) breeds, which indicates a zebu gene introgression into taurine breeds. In this

pirenaica (taurine) study, the locus BM2113 showed the zebu diagnostic 131-bp allele with a frequency of 0.149. In addition, other zebu diagnostic alleles were observed at very low frequencies such as ETH152-193 (0.075) and BM2113-123 (0.009). Zebu and African-type STR alleles have previously been reported in Iberian cattle [37,38], while the African mitochondrial T1 haplogroup was also observed in Pirenaica [39]. Therefore, zebu markers may suggest a North African genetic signature in Pirenaica cattle, based on the hypothesis of Neolithic dispersal through the Mediterranean route and historical migrations [40,41].

The average heterozygosity value (0.680) was similar to the value reported in Pirenaica by Rendo et al. [14] using 11 STRs (0.688) and higher than the value reported by Cañon et al. [13] using 16 STRs (0.628). These two studies had smaller sample sizes. In contrast, Martin-Burriel et al. [42] showed the lowest He in Pirenaica, probably due to sampling or panel selection even 30 STR were genotyped. Overall, average heterozygosity value was slightly higher than values reported in native breeds from Spain, Portugal and France ranging from 0.50 to 0.71 [6,13].

The PIC values per locus varied between 0.302 and 0.845, with a mean value of 0.637. A PIC value exceeding 0.5 indicates highly polymorphic microsatellite marker, while values ranging from 0.25 to 0.5 indicate medium-polymorphic loci. In Pirenaica, the PIC values of most loci exceeded 0.6, except for HEL13 (0.421), INRA035 (0.384) and ILST005 (0.302). Based on heterozygosity and PIC values, Pirenaica had slightly lower genetic diversity than other Basque breeds such as Terreña, Monchina and Betizu [14,15]. According to Mendizabal et al. [43], the Pirenaica population increased significantly during the 1850s, but the later introgression of new cattle breeds from Europe lead to an endangered situation of the former breed. It was not until 1975, when the need to maintain sustainable production systems using native animal genetic resources promoted the recovery and improvement of Pirenaica. At present, Pirenaica has the largest population size compared to other aforementioned native Basque breeds and it is the first native cattle breed being included in a selection program in the Basque region. Therefore, the selection from a reduced number of reproducers might imply lower heterozygosity and a reduction of the number of alleles in comparison with aforementioned native breeds that have been kept in a semi wild natural environment. In fact, this study has showed a smaller mean number of alleles per locus (7.54 ± 2.5) in Pirenaica compared to Betizu (7.91 ± 2.51) or Terreña (8.64 ± 2.54) from a previous study [14]. However, our Pirenaica sample showed a higher mean number of alleles per locus than others, which ranged between 6.22 and 6.91 [12,14].

The relationship between Pirenaica and other breeds produced in the Basque region was studied (Figure 1) as relevant for the overall knowledge related to cattle genetic resources. Terreña (basque native breed) and other allochthonous breeds used in the region have been studied such as Salers, a rustic cattle breed used for beef production, which has grown in importance due to its ready adaptability to local management and environmental conditions. Furthermore, the Holstein-Friesian breed is primarily used as a cull dairy cow, which is also an integral part of the Basque regional beef supply chain. The exact test for population differentiation based on allele frequency showed that the breeds were significantly different from each other ($p < 0.001$). Pirenaica and terreña breeds are both native from the Basque region and showed certain admixture (Figure 1). A marked differentiation of Pirenaica was observed in the FCA plot in comparison with Salers and Holstein-Friesians (Figure 1a). These results confirm the genetic differences among native Pirenaica compared with Salers and Holstein-Friesian breeds, which have a distant geographical origin in Europe. The NJ phylogenetic tree showed three branches that mainly corresponded to Pirenaicas, Salers and Holstein-Friesians (Figure 1b). The Terreña breed was not well separated in the NJ tree, and even it has been less subjected to intensive selection. Balanced samples might be necessary for future studies of relationships among Basque native breeds. Clustering analysis using a Bayesian approach identified three underlying genetic clusters (Figure 1c). According to the probabilities of K as a log-likelihood given K clusters [32], the corresponding ΔK statistic showed that maximal ΔK occurred at K = 2. Pirenaica and Terreña breeds presented the zebu-diagnostic BM2113-131 allele, which has also been described in Salers [16]. Therefore, the ΔK method may underestimate K considering a common African genetic signature in these three breeds,

whereas the geographical origin may indicate an optimal K = 3. Finally, genetic differentiation among breeds was considered. In our case, the overall genetic differentiation among breeds (F_{ST}) was high (0.1195; Table 2), as it affects the performance of assignment tests. Genetically divergent breeds ($F_{ST} > 0.1$) are more likely to be correctly assigned than closely related ones ($F_{ST} < 0.05$) [6].

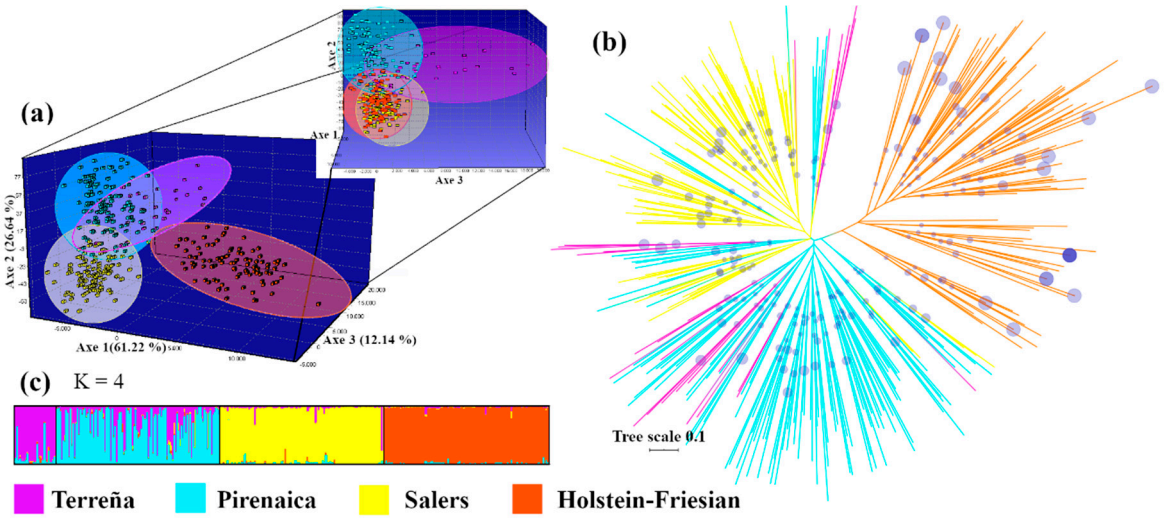

Figure 1. Genetic variations and phylogenetic differentiation analyses among cattle breeds. (**a**) 3D-FCA, (**b**) NJ radiation tree (Scale measured in R_{ST} distance values), size of circles represents bootstraps percentage and (**c**) mean probabilities of individual cluster memberships using STRUCTURE (K = 4).

Table 2. Pairwise F_{ST} (below diagonal) and R_{ST} (above diagonal) between breeds of cattle.

	Terreña	Pirenaica	Salers	Holstein-Friesian
Terreña	-	0.04277	0.14579	0.14030
Pirenaica	0.04102	-	0.11512	0.14115
Salers	0.12724	0.10324	-	0.24822
Holstein-Friesian	0.12304	0.12369	0.19886	-

All F_{ST} and R_{ST} values are significant ($p < 0.001$).

3.2. Individual Identification and Parentage Determination

In Pirenaica, the CMP value was 1.89×10^{-25} when 30 markers were used. Whereas, ISAG core panel of 12 STRs available in Genotype Panel 1.2 (Thermo Scientific) showed a CMP value of 2.3×10^{-11}. The CMP value was 3.4×10^{-13} when the most polymorphic (highest PIC value) 12 markers were selected. The present study, using 12 STR core panel or using the most polymorphic 12 STRs, showed stronger discrimination power than previous studies [44,45]. Bovine Genotype Panel 2.2 (Thermo Scientific), which includes six additional STRs, could not be completely evaluated since three of its STRs (MGTG4B, RM67 and SPS113) were not studied following the recommendations [10]. However, laboratories that perform bovine parentage analyses usually use other complementary panels when more discrimination power is required for resolving complicated parentage cases.

In paternity testing, PE for each locus was measured as the probability of excluding an individual (sire) from being the father of the calf. PE was computed considering one known parent (PE-1P, dam not typed and random sire matched against calf) or two known parents (PE-2P, random sire matched against dam/calf pairs). In Pirenaica, the CPE for PE-1P and PE-2P was computed considering all 30 loci, but also considering the reduced loci number used in reference studies. Pirenaica showed similar CPE_1 (0.9911; Figure 2) to Terreña (0.9918) when 11 markers were used [15], while CPE_2 was 0.9997 in both breeds. When using the minimum 12-STR core panel recognized by ISAG, Pirenaica had a CPE_1 and CPE_2 value of 0.9946 and 0.9998, respectively. In other European breeds, 12-STR panel showed CPE_1 values that ranged from 0.9135 to 0.9777 and CPE_2 values that ranged from 0.9935 to

0.9999 [11]. However, a CPE value over 99.99% is necessary for paternity analysis, and, therefore, the 12-STR panel might be insufficient depending on breed and parentage. This evidenced the need to increase the number of STR loci in Pirenaica, in order to have enough exclusion power to resolve satisfactorily parentage cases. Van de Goor [11] also considered a 16-STR panel in several European bovine breeds showing a CPE_1 values ranging from 0.9818 to 0.9994, and a CPE_2 values from 0.9935 and 0.9999. However, the Pirenaica was not included in their forensic study.

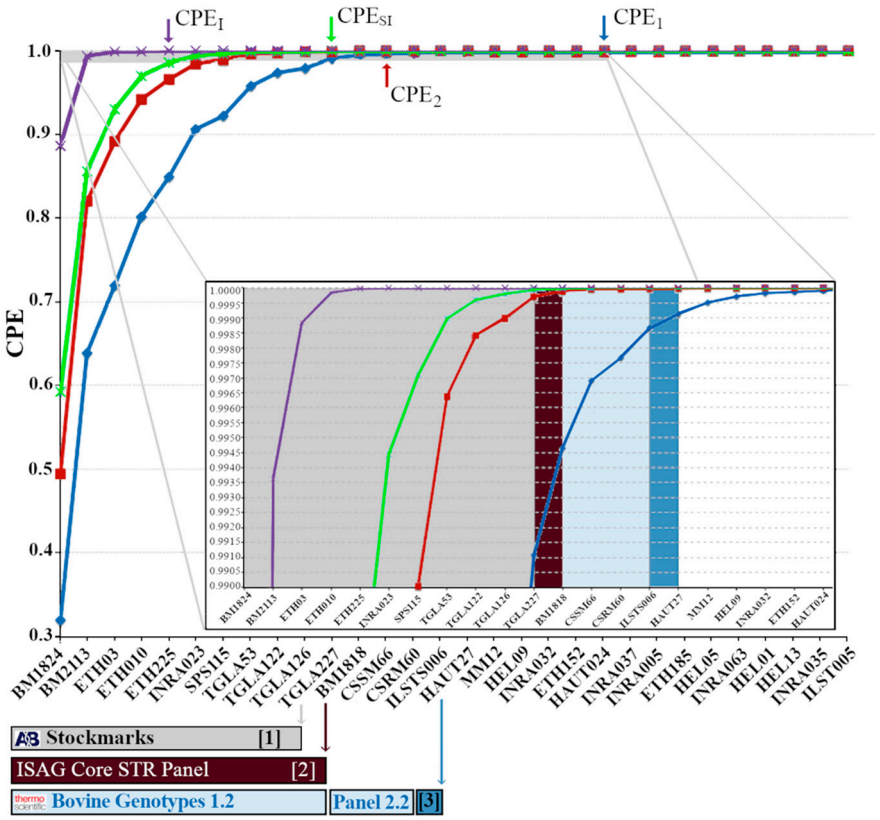

Figure 2. Comparison of combined power of exclusions (CPE) between the panels of short tandem repeat markers. Short tandem repeats (STRs) considering one known parent, two known parents, sibling and identical are shown in blue (CPE_1), red (CPE_2), green (CPE_{SI}), and purple ($CPE_I = 1 - CMP$) cases, respectively. Little arrows (top) show the minimal number of markers for 99.99% of CPE. Boxes (below) show the markers included in recommended and commercial STR panels (grey, Stockmarks; garnet, ISAG core; blue, Bovine genotype 1.2 and 2.2). Numbers in brackets shows other panels used in literature.

The recommended 30-STR panel, used in this study, showed a CPE_1 (considering one known parent) and CPE_2 (two known parents) values of 0.99998 and 0.99999997, respectively. Our Pirenaica results showed that a 21-STR panel might be necessary to obtain a CPE_1 value of 0.9999, while a 13-STR panel is enough for a discriminative CPE_2 value of 0.9999 (Figure 2). Finally, for a sibling analysis in the Pirenaica breed, a 11-STR panel is enough for a CPE_{SI} value of 0.9999, which is similar to the values observed in other European breeds [11]. Overall, for most forensic cases, except CPE_{SI}, the 12-STR core panel seems insufficient in Pirenaica and an increase in the amount of genotyped STRs should be considered. A 21-STR panel looks more appropriate to resolve some parentage analysis in Pirenaica.

3.3. Assignment Analysis of Breeds

In the assignments of GENECLASS, over 99% of individuals were allocated within their populations using both frequency (99.67%) and Bayesian (99.70%) methods using 30 STRs. Previous studies showed

an assignment success between 67% and 100% using 6 to 23 STR data from 4 to 7 cattle breeds [6,44]. However, the assignment method and the confidence of the test should be considered since they greatly influence the assignment success. Genetic differentiation among breeds is also of interest as it affects the performance of assignment tests; genetically divergent breeds ($F_{ST} > 0.1$) are more likely to be correctly assigned than closely related ones ($F_{ST} < 0.05$) [6]. In our case, the mean F_{ST} among breeds was also slightly higher (Table 2) than the aforementioned studies. Only low differentiation in allele frequencies was observed between Pirenaica and Terreña native breeds ($F_{ST} < 0.1$).

In the Bayesian assignment of STRUCTURE, a study, using the same 30 STRs recommended by ISAG-FAO, showed an assignment between 89.3% and 95.8% in Korean native cattle [46]. In this study, considering the 30 STRs panel and a cutoff value of 80% ($Q \geq 0.8$), the overall proportion of animals correctly assigned to a breed was 98.8%. However, assignment tests were performed using a 11 STR panel and Basque native Terreña genetic data [15]; Pirenaica assignment decreased to 66.1%, whereas 31% of Pirenaica animals were uncorrected assigned to Terreña breed. In contrast, the Terreña, Salers and Holstein-Friesian showed higher assignments, 85.3, 94.8 and 96.6%, respectively. Therefore, a STRUCTURE assignment test suggests that the number of STRs should be increased when other native breeds are included. Although previous methods (GENECLASS or STRUCTURE) have been extensively used for the assignment, they encounter several limitations. The frequency-based method lacks the *p* value for measuring the confidence with which an individual belongs to a given population. Whereas, previous Bayesian methods can bias assignments or provide inaccurate results if sample sizes are unbalanced among populations [35,47]. In order to overcome the problem of unbalanced population sizes, a machine-learning approach (assignPOP package, R software) was applied to study the mean and variance of assignments (Figure 3). This approach combines various markers panels into a single predictive model, not possible in previous methods, while it can provide the minimum number and most discriminative markers necessary for accurate assignments. Firstly, Pirenaica was assessed against the 11-STR panel used in the Terreña breed [15] (Figure 3a). The means of assignment were generally low in Pirenaica (62.9%) and Terreña (58.2%), while high variance was observed in the bar-plots due to the non-accuracy of the assignment tests. In general, the 11-STR panel might not be enough for an accurate assessment of an individual to its population of origin, when geographically and phylogenetically related native Pirenaica and Terreña breeds are studied. A 16-STR panel reported in Blonde d´Aquitaine and Limousin [11], breeds also included in the Basque PGI label, was used against Pirenaica (Figure 3b). High mean assignment value was observed in Pirenaica (98.9%) in comparison to Blonde d'Aquitaine (45.4%) and Limousin (54.1%) considering balanced training sets of 25, 50 and 75 individuals. The overall mean assignment was of 66.1%, which increases with the amount of STRs included. In our study, lower assignments in Blonde d'Aquitaine and Limousin should be carefully considered since kinship is unknown in these breeds' data. These two breeds are native from the south of France, a close Basque region where they have also been traditionally raised, and therefore they are included in the local PGI. However, future sampling and genotyping of Blonde and Limousin grown in the Basque region could obtain more trustable assignments along with Pirenaica.

Finally, several subpanels (11, 16 and 21 STRs) and a 30-STR panel assignment were compared (Figure 3c). Pirenaica and Salers showed over 90% mean assignments for the 16, 21 and 30-STR panels, while percentages were lower for the 11-STR panel with 70.4% and 83.5%, respectively. In contrast, for Holstein-Friesians a 21-STR panel was required to reach an assignment of 90% probably related to a reduced genetic diversity promoted by its selection for milk production. In general, 11 or 12-STR commercial panels seem to be insufficient for reliable assignment tests even selecting highest F_{ST} markers. Pirenaica showed that a panel of over 21-STRs is necessary for trustworthy assignments (≥95%; Table S1). In essence, a reliable molecular traceability to ensure a correct assessment of an unknown beef product to its origin PGI label will depend on a well-designed STR panel containing the minimum amount of STRs with the highest F_{ST} values.

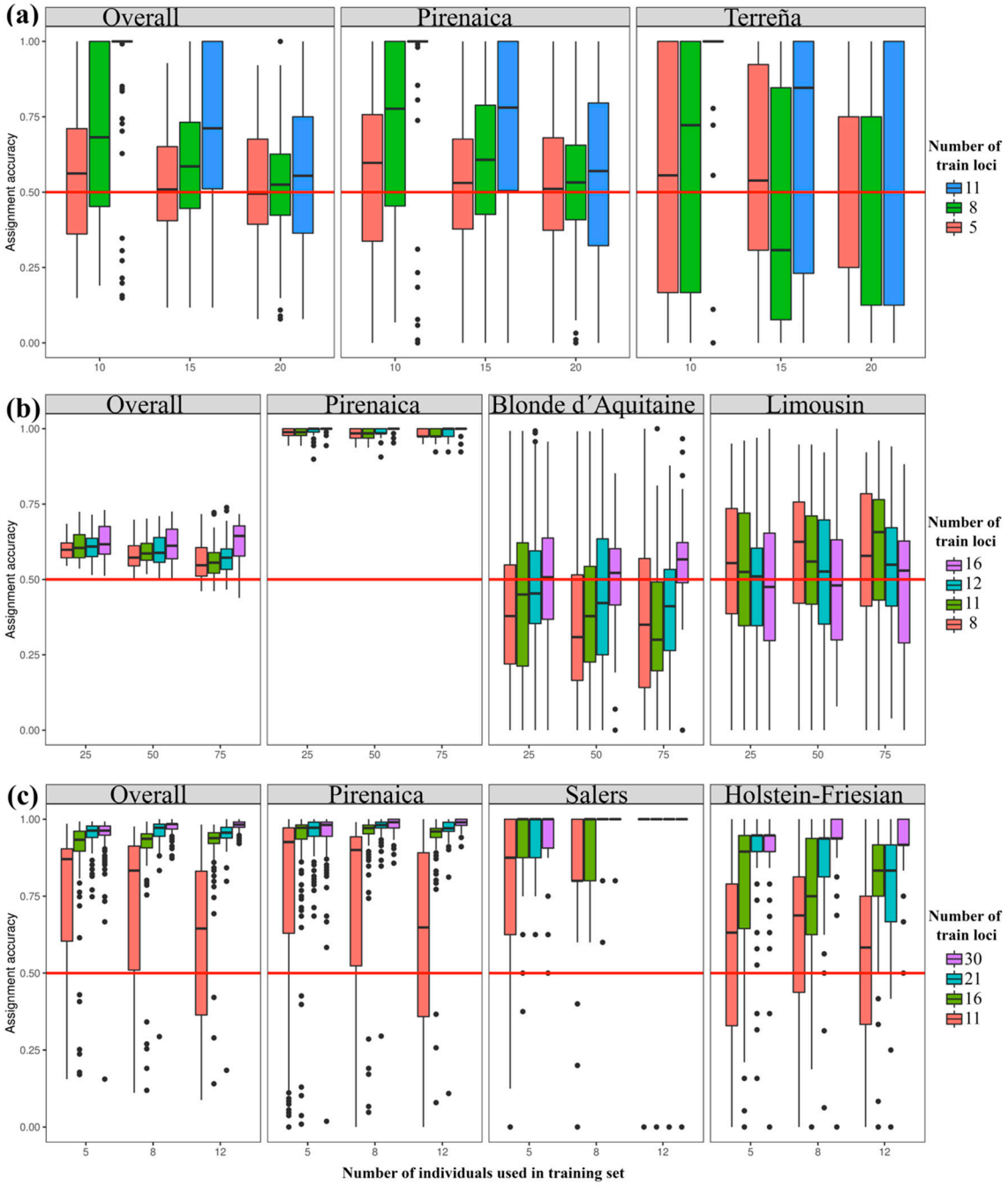

Figure 3. Assignment accuracies (%), according to Monte-Carlo cross-validation and depicted as bar-plots. (**a**) Pirenaica and Terreña breeds' assignments for balanced populations (10, 15 and 20 individuals) crossed by three levels of train STRs with the highest F_{ST} (red: 5-STR; green: 8-STR) and all loci (blue: 11-STR), (**b**) Pirenaica, Blonde d´Aquitaine and Limousin breeds' assignments for balanced populations (25, 50 and 75 individuals) crossed by four levels of train STRs with the highest F_{ST} (red: 8-STR; green: 11-STR; turquoise: 12-STR) and all loci (fuchsia: 16-STR). (**c**) Pirenaica, Salers and Holstein-Friesian breeds' assignments for balanced populations (5, 8 and 12 individuals) crossed by four levels of train STRs with the highest F_{ST} (red: 11-STR; green: 16-STR; turquoise: 21-STR) and all loci (fuchsia: 30-STR).

Up to date parentage control in cattle has been mainly based on STR but it is currently moving towards single nucleotide polymorphism (SNP)-based methods [48]. STRs are highly polymorphic and spread throughout the entire genome [49]. However, its analyses are time consuming, even for

trained staff, due to the inconsistencies in allele size calling and errors in size determination by different laboratories. On the other hand, even SNPs are biallelic markers, high-throughput sequencing has permitted the development of high-density SNP panels with sufficient power to uniquely identify individuals. These SNP panels have increasing advantages such as greater abundance, genetic stability, simpler nomenclature and manageable to automated analysis [50]. In fact, a core panel of 100 and 100 additional SNPs have been defined for parentage control [51]. The cost per SNP is low compared to microsatellites, but the cost of the high-density assays might be prohibitive for many applications and the equipment necessary for high-throughput SNP panels is still quite expensive [10]. In contrast, in the present study, it is estimated a cost of EUR 10–20/sample for multiplex STR genotyping is needed, which makes this approach affordable for routine traceability in the food supply chain. Moreover, in case of the Pirenaica breed, the heterozygosity and the availability of large databases of STRs in cattle herd books, supposes a considerable reason to keep using these polymorphisms although SNPs will also provide promising advantages that need further research to evaluate the traceability and forensic effectiveness of an SNP panel in the Pirenaica breed.

4. Conclusions

The population data presented in this study provide extensive information regarding the discrimination power of a 12-STR ISAG core panel compared with the 30-STR complete panel. It has been demonstrated that the 30-STR panel is necessary as a first step to select the most appropriate markers in Pirenaica breed. In fact, the 21-STR panel is necessary for paternity analyses reaching in most cases a CPE_1 value over 99.99%. Assignment tests using subpanels and some commercial panels are insufficient and the number of markers should also be increased for reliable assignments in Pirenaica and other breeds typically used for beef production in the Basque region. Overall, the present study recommends a new assignment approach by selecting the most polymorphic markers in order to design an appropriate STR panel to increase its discrimination power at a reduced time and cost of analysis.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2615/10/9/1584/s1, Table S1: Microsatellite markers ordered according to the hightest F_{ST} value.

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animals

Supplementary Materials: Genetic Characterization of the Local Pirenaica Cattle for Parentage and Traceability Purposes

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Table S1. Microsatellite markers ordered according to the highest F_{ST} value in Pirenaica breed.

Ranking	Marker	F_{ST}
1	ILST005	0.289
2	HEL05	0.195
3	ETH185	0.177
4	TGLA126	0.159
5	BM2113	0.146
6	HAUT27	0.136
7	INRA037	0.136
8	TGLA122	0.134
9	HAUT024	0.129
10	CSSM66	0.121
11	SPS115	0.120
12	HEL13	0.118
13	INRA063	0.116
14	ETH225	0.115
15	ETH152	0.103
16	HEL09	0.094
17	TGLA53	0.086
18	ETH010	0.073
19	ILSTS006	0.066
20	MM12	0.057
21¶	INRA023	0.051
22	ETH03	0.046
23	INRA032	0.046
24	TGLA227	0.045
25	INRA035	0.031
26	BM1818	0.023
27	CSRM60	0.018
28	HEL01	0.016
29	INRA005	0.012
30	BM1824	0.002

A minimum of 21 markers are necessary for trustworthy assignments (≥ 95 %) in Pirenaica breed.

APPENDIX IV.

PUBLICATION IV.

Gamarra, D., Aldai, N., Arakawa, A., Barron, L., López-Oceja, A., de Pancorbo, M. M., & Taniguchi, M. (2018). Distinct correlations between lipogenic gene expression and fatty acid composition of subcutaneous fat among cattle breeds. *BMC veterinary research*, *14*(1), 167.

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RESEARCH ARTICLE Open Access

Distinct correlations between lipogenic gene expression and fatty acid composition of subcutaneous fat among cattle breeds

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Abstract

Background: The fatty acid (FA) composition of adipose tissue influences the nutritional quality of meat products. The unsaturation level of FAs is determined by fatty acid desaturases such as stearoyl-CoA desaturases (SCDs), which are under control of the transcription factor sterol regulatory element-binding protein (SREBP). Differences in *SCD* genotype may thus confer variations in lipid metabolism and FA content among cattle breeds. This study investigated correlations between FA composition and lipogenic gene expression levels in the subcutaneous adipose tissue of beef cattle breeds of different gender from the Basque region of northern Spain. Pirenaica is the most important beef cattle breed in northern Spain, while Salers cattle and Holstein-Friesian cull cows are also an integral part of the regional beef supply.

Results: Pirenaica heifers showed higher monounsaturated FA (MUFA) and conjugated linoleic acid (CLA) contents in subcutaneous adipose tissue than other breeds ($P < 0.001$). Alternatively, Salers bulls produced the highest oleic acid content, followed by Pirenaica heifers ($P < 0.001$). There was substantial variability in *SCD* gene expression among breeds, consistent with these differences in MUFA and CLA content. Correlations between *SCD1* expression and most FA desaturation indexes (DIs) were positive in Salers ($P < 0.05$) and Pirenaica bulls, while, in general, *SCD5* expression showed few significant correlations with DIs. There was a significant linear correlation between *SCD1* and *SRBEP1* in all breeds, suggesting strong regulation of *SCD1* expression by *SRBEP1*. Pirenaica heifers showed a stronger correlation between *SCD1* and *SREBP1* than Pirenaica bulls. We also observed a opposite relationship between *SCD1* and *SCD5* expression levels and opposite associations of isoform expression levels with the Δ9 desaturation indexes.

Conclusions: These results suggest that the relationships between FA composition and lipogenic gene expression are influenced by breed and sex. The opposite relationship between *SCD* isoforms suggests a compensatory regulation of total SCD activity, while opposite relationships between *SCD* isoforms and desaturation indexes, specially 9*c*-14:1 DI, previously reported as an indicator of SCD activity, may reflect distinct activities of SCD1 and SCD5 in regulation of FA content. These findings may be useful for beef/dairy breeding and feeding programs to supply nutritionally favorable products.

Keywords: Cattle, Desaturation index, Fatty acid composition, Gene expression, Lipid metabolism, *SCD*, Subcutaneous adipose tissue, *SREBP*, Δ9-desaturase

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Background

In recent years, consumers have expressed growing concern regarding the amount and types of dietary fat due to reported deleterious health effects of saturated and *trans* fatty acids (FAs) [1]. On the other hand, monounsaturated FAs (MUFAs) and polyunsaturated FAs (PUFAs) are recognized as beneficial for human health [2]. The FA composition of meat influences the lipid melting point [3], and an increase in the ratio of MUFA to saturated FA (SFA) increases fat softness, thereby improving palatability. Thus, enhancing MUFA content improves both the quality and nutritional value of animal products [4].

Pirenaica is the most important beef cattle breed raised in the Basque region of northern Spain and is highly appreciated both for its value as a genetic resource as well as for the production system that has developed around it. In addition to Pirenaica, Salers, a rustic cattle breed used for beef production, has grown in importance due to its ready adaptability to local management and environmental conditions [5]. Finally, Holstein-Friesian, primarily as cull dairy cows, are also an integral part of the regional beef supply chain.

There have been a number of studies investigating associations between lipogenic enzyme genotype and FA composition in cattle. In the subcutaneous and intramuscular fat depots of beef cattle, the majority of SFA conversion to MUFA is catalyzed by stearoyl-CoA desaturase (SCD, EC 1.14.19.1 or Δ9-desaturase) [6]. In addition, SCD enzyme can also catalyze the conversion of substrates like vaccenic acid (11 *t*-18:1) to its corresponding conjugated linoleic acid (CLA) isomer (9*c*,11 *t*-18:2 or rumenic acid). The association of *SCD*1 genotype with FA composition has been previously investigated in Japanese Black [7], Canadian Holstein [8], Fleckvieh [9] and crossbred cattle [10]. In addition to regulation of FA profile by *SCD1*, the novel Δ9-desaturase isoform *SCD5*, previously found in humans, has also been identified in cattle which shares 65% identity at the amino acid level [11]. Further, a relationship between genetic polymorphisms in *SCD5* and the ratio of SFA to unsaturated FA (UFA) has been reported in Holstein milk fat [12]. It thus appears that both bovine isoforms *SCD*1 and *SCD*5 contribute to FA composition. Therefore, the mechanism by which *SCD* isoforms are activated is a major determinant of FA composition and of great interest to breeders.

Da Costa et al. [13] reported a correlation between *SCD1* expression and FA composition of subcutaneous fat in Portuguese cattle, whereas the expression of *SCD5* and its relation to the subcutaneous FA profile was not investigated. The *SCD1* gene is controlled by the key transcription factor *SREBP1* [14]. In Japanese Black beef cattle, *SREBP1* polymorphisms have been associated with FA composition [15]. Alternatively, transcriptional regulation of bovine *SCD*5 remains unclear, although a recent study using human choriocarcinoma trophoblastic cells (JEG3) reported that *SREBP1* can bind to the *SCD5* promoter [16].

The complex associations between the biochemical pathways regulating fat content and genetic variability of lipogenic genes are not yet fully understood in European cattle breeds, although recent studies have begun to elucidate these relationships in a specific genetic background of Japanese Black cattle [17]. The objectives of the present study were to investigate the expression levels of three key genes controlling Δ9-desaturated FA content, *SCD1*, *SCD5*, and *SREBP1*, and their associations with the FA composition of subcutaneous adipose tissue from the major commercial cattle breeds produced in northern Spain, Pirenaica, Salers, and Holstein-Friesian. Based on the findings of this study, we discuss how these associations may give information on the mechanisms of the differences in meat quality among these cattle commercial types.

Methods

Sample collection

In the present study, cattle commercial types typically destined for meat production in northern Spain (Basque region) were examined. Sample collection was designed according to data from the Bovine Identification Document and inferred relationships (parentage and sibships) computed from 29 microsatellites (Software Colony 2.0.6.2) [18]. Neither parentage nor maternal half-sibs were observed, and paternal half-sibs were maintained at low frequencies (Pirenaica, 0.009; Salers 0.013; Holstein-Friesian, 0.019). A total of 100 subcutaneous adipose tissue samples were collected from pure breed cattle (13 Salers bulls, 37 Pirenaica bulls, 29 Pirenaica heifers, and 21 Holstein-Friesian) slaughtered in a local commercial abattoir (Urkaiko S. Coop., Zestoa, Gipuzkoa, Spain) during 12 days over 5 weeks in June and July 2014. Animals came from different farms [19].

Backfat samples were obtained from the left half carcass between the 5-6th ribs and stored in plastic bags with the air removed for FA analysis or preserved in RNAlater™ (Ambion, Austin, TX) for RNA analysis. All samples were transported to the laboratory in insulated coolers and stored at − 80 °C until analysis.

Salers and Pirenaica were yearling calves with similar age (average of 12.9 ± 1.4 months), while Holstein-Friesian were cull cows (70.0 ± 19.43 months) at slaughter, which are regular ages of commercial types used for beef production in the region. Hot carcasses of Salers and Pirenaica commercial bulls were of similar weight (average of 325 ± 38.4 kg) while carcasses of Pirenaica

heifers and Holstein-Friesian cows were markedly lighter (291 ± 59.6 and 253 ± 33.1 kg, respectively).

In the abattoir, conformation and degree of fat cover of each carcass were recorded. European regulations were followed for carcass classification at 24 h post-mortem [20] including the EUROP scale for conformation and a 1-to-5 scale for fat cover scoring. Each level of both scales was divided in 3 sub-levels and transformed to a numerical scale ranging from 1 to 15, with 15 being the best conformation and the thickest fat cover.

Fatty acid composition

A 50 mg sample of subcutaneous fat tissue was weighed, freeze-dried, and directly methylated with sodium methoxide (0.5 N methanolic base, Supelco Inc., Bellefonte, PA, USA) [21]. For quantitation, 1 mL of internal standard (23:0 methyl ester) was added prior to methylation, and FA methyl esters were analyzed by gas chromatography with flame ionization detection (GC/FID) using two complementary 100 m columns (SP-2560 [22] and SLB-IL111 [23]) and following the conditions and details reported in [24]. Main FA groups and potential Δ9 substrates, products, and inhibitors (10 *t*,12*c*-18:2; [25]) have been determined for the individual FAs measured in this study. From the potential substrates (14:0, 15:0, 16:0, 17:0, 18:0, 19:0, 20:0, 6-8 *t*-18:1, 11 *t*-18:1, 12 *t*-18:1, 13 *t*/14 *t*-18:1 and 15*c*-18:1) and products (9*c*-14:1, 9*c*-15:1, 9*c*-16:1, 9*c*-17:1, 9*c*-18:1, 9*c*-19:1, 9*c*-20:1, 7 *t*,9*c*-18:2, 9*c*,11 *t*-18:2, 9*c*,12 *t*-18:2, 9*c*,13 *t*-18:2, 9*c*,15*c*-18:2), individual desaturation indexes were calculated by the following formula:

$$\text{Desaturation index (DI)} = [\text{product}]/([\text{substrate}] + [\text{product}]).$$

Total DI (sum of all individual DIs) was also computed for each commercial type, while minor products and substrates (i.e., 11 *t*,15*c*-18:2 & 9*c*,11 *t*,15*c*-18:3 [26]) or below quantification limits were not considered in the present study.

RNA extraction and quantitative real-time PCR

A 100 mg sample of frozen subcutaneous fat tissue was disrupted and simultaneously homogenized to fine powder with a mortar and pestle under liquid N_2. Total RNA was extracted using the RNeasy Lipid Tissue kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's instructions. An additional DNase digestion step was performed to remove any contaminating genomic DNA. Concentration and quality of the extracted RNA were assayed by measuring the 260 nm and 280 nm absorbance using a NanoDrop ND-1000 Spectrophotometer (Peqlab, Erlangen, Germany). Absorbance ratios (260/280) of all preparations were at least 1.8. Integrity of RNA was checked by denaturing agarose gel electrophoresis. Aliquots of RNA were stored at − 80 °C and dehydrated in RNAstable 96-Well Plates (Biomatrica, San Diego, CA, USA) for long-term storage. Reverse transcription was performed in a 30 μL final reaction volume containing 250 ng total RNA, 3.3 μL RNase/DNase-free water, 5 μL of 5 × RT buffer, 1.5 μL dNTPs, 0.8 μL RNAase inhibitor, 0.8 μL random primer, and 0.8 μL high efficient ReverTra Ace reverse transcriptase (TOYOBO, Osaka, Japan). Cycle parameters were 30 °C for 10 min, 42 °C for 20 min, 99 °C for 5 min, and 4 °C for 5 min. Custom TaqMan Assays (Applied Biosystems, Foster City, CA, USA) were conducted to measure the relative expression levels of bovine *SCD1*, *SCD5* and *SREBP1* using the primers and FAM/TAMRA probes reported in Table 1. Each candidate gene was amplified in multiplex with an internal control (18S rRNA Endogenous Control VIC/TAMRA Probe, Primer Limited) by the co-application reverse transcription method (Co-RT) [27]. This multiplexing approach guarantees the same conditions (thus equal amplification efficiency) and same reverse transcriptase activity for both genes, thereby yielding better normalization and reproducibility. The reaction mixture included primers (10 μM each), FAM-labeled probe (10 μM), 0.6 μL of 18S RNA Endogenous Standard containing VIC-labeled probe and limited primers, and 2 × TaqMan Gene Expression Master Mix (7.5 μL) (Applied Biosystems). Real-time PCR was performed in triplicate using the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with a standard two-step cycling program of 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The average of the gene expression levels was used for further analyses. PCR efficiency was monitored by the increase in absolute fluorescence [28], mainly because this allows PCR efficiency calculation for individual samples/reactions and prevents problems arising from the use of standard curves. Raw data were obtained from the ABI Prism 7500 SDS software v1.4, exported in Rn format, and imported to LinRegPCR (Heart Failure Research Center, Amsterdam, the Netherlands). LinRegPCR determines baseline fluorescence, sets a window of linearity for each amplicon, and calculates the PCR efficiency (E) per sample and amplicons using an iterative algorithm. In this study, efficiencies were over 90% for all samples and correlation coefficients were higher than 0.99.

The comparative threshold cycle method (ΔCt) was employed to calculate relative gene expression based on the following formula:

$$\Delta Ct = \left(Ct_{\text{target gene}} - Ct_{\text{18S rRNA gene}}\right).$$

Table 1 Primer sequences, product sizes, and annealing temperatures of bovine genes analyzed by RT-PCR

Gene symbol [GenBank accession]	Primer sequence (5′ - 3′)	Product (bp)	Annealing temperature
SCD1	P: CCTCTGGAACATCACCAGCTTCTCGGC	106	60
[NM_173959]	F: GCTGTCAAAGAAAAGGGTTCCAC		
	R: AGCACAACAACAGGACACCAG		
SCD5	P: CAGAACCCGCTCGTCACCCTGGG	82	60
[NM_001076945]	F: CCCTATGACAAGCACATCAGCC		
	R: GATGGTAGTTATGGAAACCTTCACC		
SREBP1	P: CAGCCCCAGTCCTGGATCAGCCGA	83	60
[NM_001113302]	F: CTTGGAGCGAGCACTGAATTG		
	R: GGGCATCTGAGAACTCCTTGTC		

P = probe, *F* forward, *R* reverse

Statistical analysis

Statistical analysis was conducted using IBM SPSS Statistics 22 for Windows (SPSS Inc., IBM Corporation, NY, USA). First of all, data was checked for normality and homoscedasticity. Then, the following general linear model $Y_{ijk} = \mu + CT_i + A_j + HCW_k + e_{ijk}$ was used for analysis of variance (ANOVA), including commercial type (CT; Salers bulls, Pirenaica bulls, Pirenaica heifers, Holstein-Friesian cows) as fixed effect and age at slaughter (A) and hot carcass weight (HCW) as covariates. The effect of sire was also checked but not included in the model as it was statistically not significant. LSD post hoc test was applied for multiple comparison of means among commercial breeds studied.

Simple linear regression analyses were also performed to investigate the relationship between genes (gene-gene) for each commercial type studied.

Finally, partial Pearson correlations coefficients adjusted for A and HCW were computed to determine the associations among gene expression (ΔCt) and FA (Δ9 DIs) data.

Three significant figures were used to express the data, and significance was declared at $P < 0.05$.

Results

Carcass traits and fatty acid composition

Pirenaica heifer carcasses showed the highest fat cover, while those from Pirenaica bulls and Holstein-Friesian cows were lower, and carcasses from Salers showed an intermediate degree of fat cover ($P < 0.001$). In terms of FA composition, several significant differences in specific SFA species were found among commercial types (i.e., 14:0, 16:0, 19:0, 20:0 SFAs), but there were no significant differences in total SFA content. Pirenaica heifers exhibited the highest content of *cis*- and *trans*-MUFAs, and Salers bulls had higher *cis*-MUFA content than Pirenaica bulls ($P < 0.001$; Table 2). Accordingly, Pirenaica heifers showed the highest contents of 9*c*-14:1 and 9*c*-16:1, while 9*c*-17:1 and 9*c*-18:1 were the highest in Pirenaica heifers but also in Salers bulls. Additionally, Pirenaica heifers exhibited the highest content of individual *trans*-18:1 isomers, while vaccenic acid (11 *t*-18:1) and *trans*-12-octadecenoic acid (12 *t*-18:1) contents did not differ among commercial types. The total CLA content was highest in Pirenaica heifers ($P < 0.01$). However, no significant differences were observed in rumenic acid (9*c*,11 *t*-18:2), the major CLA isomer. The second major CLA isomer (7 *t*,9*c*-18:2), other non-conjugated dienes (6-8 *t*-18:1and 13 *t*/14 *t*-18:1), and potential products of Δ9-desaturation (9*c*,12 *t*-18:2, 9*c*,13 *t*-18:2 and 9*c*,15*c*-18:2) were significantly higher in Pirenaica heifers than in the other commercial types. In contrast, n-6 PUFA content was similar in Pirenaica heifers and Pirenaica bulls, and significantly lower in both compared to Salers bulls. Finally, the content of 10 *t*,12*c*-CLA, reported as an inhibitor of Δ9-desaturase, was higher in fat tissues of Pirenaica heifers than in other commercial types ($P < 0.001$; Table 2).

Gene expression

The relative mRNA expression levels of the lipogenic genes *SREBP1*, *SCD1*, and *SCD5* were similar in Pirenaica bulls and heifers (Fig. 1). Overall, *SCD1* expression was higher than *SREBP1* expression ($P < 0.001$) and *SCD5* expression ($P < 0.001$) in all commercial types, with average−ΔCt values of − 7.91, − 13.4, and − 17.2, respectively (Fig. 1). Differences among breeds were observed for each gene. The mRNA expression of *SCD1* was significantly higher in Salers (− 7.36) and Pirenaica cattle (average −ΔCt value of − 6.10) than Holstein-Friesian cows (− 13.8) ($P < 0.001$). In contrast, *SCD5* mRNA expression was lowest in Pirenaica bulls and heifers (average of − 17.8) among commercial types, highest in Holstein-Friesians cows (− 15.3), and at intermediate expression levels in Salers bulls (− 17.1; $P < 0.001$). In addition, expression of *SREBP1* mRNA was higher in Pirenaica bulls and heifers (average of − 12.73) than in the other commercial types (average − 14.8; $P < 0.001$).

Table 2 Comparisons of fatty acid composition (mg/g of subcutaneous fat) and carcass parameters among commercial types

	Commercial type				
	Salers bulls ($n = 13$)	Pirenaica bulls ($n = 37$)	Pirenaica heifers ($n = 29$)	Holstein-Friesian cows ($n = 21$)	*p*-value
Conformation	8.45 ± 0.37^{b}	10.9 ± 0.3^{a}	11.0 ± 0.3^{a}	2.02 ± 0.64^{c}	< 0.001
Fatness	5.79 ± 0.46^{b}	4.52 ± 0.33^{c}	7.47 ± 0.33^{a}	1.96 ± 0.81^{d}	< 0.001
14:0 [s1]	31.6 ± 2.4^{ab}	30.5 ± 1.7^{b}	35.6 ± 1.8^{a}	16.0 ± 4.2^{c}	< 0.001
15:0 [s2]	4.71 ± 0.33	4.07 ± 0.23	3.99 ± 0.24	3.13 ± 0.57	0.111
16:0 [s3]	230 ± 11^{ab}	217 ± 8^{b}	246 ± 8^{a}	170 ± 20^{bc}	0.002
17:0 [s4]	9.01 ± 0.71	7.48 ± 0.50	8.08 ± 0.51	5.54 ± 1.22	0.057
18:0 [s5]	131 ± 11	119 ± 8	98.0 ± 8.4	129 ± 20	0.059
19:0 [s6]	0.565 ± 0.074^{a}	0.595 ± 0.052^{a}	0.38 ± 0.05^{b}	0.708 ± 0.129^{a}	0.005
20:0 [s7]	0.907 ± 0.119^{ab}	0.816 ± 0.084^{b}	0.468 ± 0.085^{c}	1.34 ± 0.21^{a}	< 0.001
9*c*-14:1 [p1]	8.05 ± 1.27^{b}	7.80 ± 0.90^{b}	12.4 ± 0.9^{a}	1.46 ± 2.21^{c}	< 0.001
9*c*-15:1 [p2]	0.208 ± 0.028	0.183 ± 0.020	0.206 ± 0.020	0.15 ± 0.05	0.572
9*c*-16:1 [p3]	36.7 ± 3.7^{b}	33.5 ± 2.6^{b}	45.3 ± 2.7^{a}	9.74 ± 6.51^{c}	< 0.001
9*c*-17:1 [p4]	6.73 ± 0.44^{a}	5.24 ± 0.31^{b}	6.96 ± 0.32^{a}	2.15 ± 0.77^{c}	< 0.001
9*c*-18:1 [p5]	308 ± 14^{a}	261 ± 10^{b}	333 ± 10^{a}	196 ± 24^{c}	< 0.001
9*c*-19:1 [p6]	0.987 ± 0.055^{a}	0.789 ± 0.039^{b}	0.801 ± 0.040^{b}	0.795 ± 0.096^{ab}	0.004
9*c*-20:1 [p7]	0.726 ± 0.075	0.614 ± 0.053	0.728 ± 0.054	0.777 ± 0.130	0.231
6-8 *t*-18:1 [s8]	3.14 ± 0.38^{bc}	3.66 ± 0.27^{ab}	4.12 ± 0.28^{a}	1.83 ± 0.67^{c}	0.010
11 *t*-18:1 [s9]	10.3 ± 2.0	12.1 ± 1.4	8.20 ± 1.47	6.15 ± 3.55	0.162
12 *t*-18:1 [s10]	2.26 ± 0.28	2.56 ± 0.20	2.71 ± 0.21	1.59 ± 0.50	0.174
13 *t*/14 *t*-18:1 [s11]	4.37 ± 0.51^{b}	5.23 ± 0.36^{ab}	5.80 ± 0.37^{a}	3.67 ± 0.89^{ab}	0.029
15*c*-18:1 [s12]	1.08 ± 0.16^{c}	1.38 ± 0.11^{b}	2.07 ± 0.11^{a}	0.596 ± 0.271^{c}	< 0.001
7 *t*,9*c*-18:2 [p8]	0.813 ± 0.116^{bc}	0.845 ± 0.082^{b}	1.25 ± 0.08^{a}	0.295 ± 0.201^{c}	< 0.001
9*c*,11 *t*-18:2 [p9]	3.11 ± 0.53	3.26 ± 0.376	3.53 ± 0.38	1.80 ± 0.92	0.453
9*c*,12 *t*-18:2 [p10]	0.520 ± 0.067^{b}	0.608 ± 0.048^{b}	0.854 ± 0.048^{a}	0.312 ± 0.118^{b}	< 0.001
9*c*,13 *t*-18:2 [p11]	0.963 ± 0.131^{b}	1.07 ± 0.09^{b}	1.61 ± 0.09^{a}	0.567 ± 0.229^{b}	< 0.001
9*c*,15*c*-18:2 [p12]	0.461 ± 0.044^{b}	0.341 ± 0.031^{c}	0.587 ± 0.032^{a}	0.266 ± 0.077^{bc}	< 0.001
10 *t*,12*c*-18:2 [i]	0.221 ± 0.041^{b}	0.195 ± 0.029^{b}	0.324 ± 0.030^{a}	0.099 ± 0.072^{b}	0.001
SFA	410 ± 21	382 ± 15	395 ± 15	328 ± 37	0.285
MUFA	427 ± 18^{b}	379 ± 13^{c}	489 ± 13^{a}	245 ± 32^{d}	< 0.001
cis-MUFA	385 ± 18^{b}	331 ± 13^{c}	430 ± 13^{a}	221 ± 32^{d}	< 0.001
trans-MUFA	42.2 ± 5.2^{bc}	48.3 ± 3.6^{b}	58.9 ± 3.7^{a}	23.5 ± 9.0^{c}	0.001
CLA	4.92 ± 0.56^{bc}	5.22 ± 0.40^{b}	6.42 ± 0.40^{a}	2.68 ± 0.98^{c}	0.002
PUFA	29.9 ± 2.1^{a}	24.3 ± 1.5^{bc}	24.4 ± 1.5^{b}	14.9 ± 3.7^{c}	0.009
n-6	27.7 ± 2.0^{a}	22.2 ± 1.4^{b}	22.1 ± 1.4^{b}	12.8 ± 3.4^{c}	0.005
n-3	2.09 ± 0.19	2.05 ± 0.14	2.15 ± 0.14	2.04 ± 0.33	0.942

Least square means ± standard deviations

t trans, *c cis*, *SFA* saturated fatty acids, *MUFA* monounsaturated fatty acids, *CLA* conjugated linoleic acid, *PUFA* polyunsaturated fatty acids

[s] substrate; [P] product; [1-12]Same superscript numbers indicate the substrate-product pairs

[i] inhibitor of SCD enzyme [25]

[a,b,c,d] Values within a row with different superscripts differ significantly at $P < 0.05$

SFA = 10:0 + 12:0 + 13:0 + 14:0 + 15:0 + 16:0 + 17:0 + 18:0 + 19:0 + 20:0 + 21:0 + 22:0 + 23:0 + 24:0

MUFA = 9*c*-14:1 + 9*c*-15:1 + 7*c*-16:1 + 9*c*-16:1 + 10*c*-16:1 + 11*c*-16:1 + 12*c*-16:1 + 13*c*-16:1 + 5*c*-17:1 + 7*c*-17:1 + 9*c*-17:1 + 9*c*-18:1 + 11*c*-18:1 + 12*c*-18:1 + 13*c*-18:1 + 14*c*-18:1 + 15*c*-18:1 + 16*c*-18:1 + 9*c*-19:1 + 11*c*-19:1 + 13*c*-19:1 + 9*c*-20:1 + 11*c*-20:1 + 6 *t*/7 *t*-16:1 + 8 *t*-16:1 + 9 *t*-16:1 + 10 *t*-16:1 + 11 *t*/12 *t*-16:1 + 4 *t*-18:1 + 5 *t*-18:1 + 6-8 *t*-18:1 + 9 *t*-18:1 + 10 *t*-18:1 + 11 *t*-18:1 + 12 *t*-18:1 + 13 *t*/14 *t*-18:1 + 15 *t*-18:1 + 16 *t*-18:1

CLA = 9*c*,11 *t*-18:2 + 7 *t*,9*c*-18:2 + 8*c*,10 *t*-18:2 + 9 *t*,11*c*-18:2 + 11*c*,13 *t*-18:2 + 10 *t*,12*c*-18:2 + 11 *t*,13*c*-18:2 + other *t*,*t*-18:2

PUFA = 18:2n-6 + 18:3n-6 + 20:2n-6 + 20:3n-6 + 20:4n-6 + 22:4n-6 + 18:3n-3 + 18:4n-3 + 20:5n-3 + 22:5n-3 + 22:6n-3 + 20:3n-9

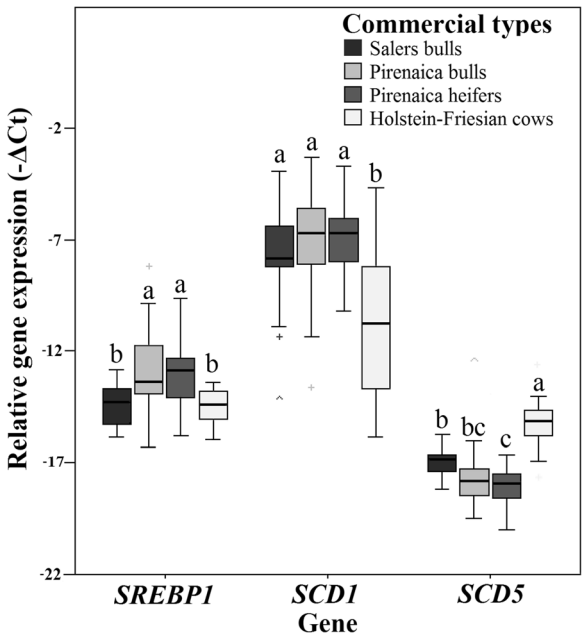

Fig. 1 Box-plot showing the relative expression levels of *SCD1*, *SCD5* and *SREBP1* in subcutaneous fat samples from the cattle commercial types Salers, Pirenaica bulls, Pirenaica heifers and Holstein-Friesian heifers. The middle line in the box represents the median, upper and lower areas of the center box indicate the 75th and 25th percentiles respectively, and vertical bars indicate standard errors. Differences among commercial types are indicated by different letters ($P < 0.05$)

Relationships among gene expression and fatty acid composition data

Significant correlations were observed between studied gene pairs in all commercial types, with particularly strong correlation between *SCD1* and *SREBP1* (Fig. 2a). Pirenaica heifers showed the highest regression coefficient between *SCD1* and *SREBP1* among the commercial types ($R^2 = 0.491$; $P < 0.001$). Salers bulls and Holstein-Friesian cows also showed relatively high regression coefficients between *SCD1* and *SREBP1* ($R^2 = 0.385$; $P = 0.024$ and $R^2 = 0.395$; $P = 0.002$, respectively), while Pirenaica bulls showed the lowest values ($R^2 = 0.239$; $P = 0.002$). A positive correlation between *SCD5* and *SREBP1* (Fig. 2b) was observed in Pirenaica bulls ($R^2 = 0.114$; $P = 0.040$) and Holstein-Friesian cows ($R^2 = 0.213$; $P = 0.035$), while in Salers bulls and Pirenaica heifers was not ($P > 0.05$). No significant correlations were observed between *SCD5* and *SCD1* gene expression except for Holstein-Friesian cows ($R^2 = 0.266$, $P = 0.017$; Fig. 2c).

In all commercial types, *SREBP*1 expression was positively correlated with the DI of most FA species, and correlations were significant for 9*c*-15:1 and 7 *t*,9*c*-18:2 in Pirenaica bulls and 9*c*-15:1 and 9*c*,12 *t*-18:2 in Pirenaica heifers ($P < 0.05$; Fig. 3a). In general, Salers bulls showed the highest positive correlations ($R > 0.65$) between *SCD*1 expression and DIs for 9*c*-16:1, 9*c*-17:1, 9*c*-18:1, 9*c*-20:1, 7 *t*,9*c*-18:2 and 9*c*,12 *t*-18:2 (Fig. 3b). Pirenaica bulls also showed significant positive correlations between *SCD*1 expression and DIs for 9*c*-17:1, 9*c*,13 *t*-18:2, and 9*c*,15*c*-18:2 DIs ($P < 0.05$), while Pirenaica heifers did not ($P > 0.05$). In contrast to *SREBP*1 and *SCD*1, there were few significant correlations between *SCD*5 and DIs among commercial types (Fig. 3c). A negative correlation was observed between *SCD5* and 9*c*,12 *t*-18:2 DI in Salers and 9*c*-14:1 DI in Pirenaica heifers ($P < 0.05$). Total DI was positively correlated with *SCD*1 in Salers ($R > 0.65$, $P < 0.05$) and Pirenaica bulls ($R > 0.35$, $P < 0.05$), but negatively correlated with *SCD*5 in Salers bulls ($R > 0.60$, $P < 0.05$).

Discussion

Fat deposition and the FA composition of fat depots are controlled by a complex regulatory system including lipogenesis and lipolysis pathways. Adipose tissue is the main site for the storage of excess energy in the form of triacylglycerols, with the Δ9-desaturase product oleic acid (9*c*-18:1) being the predominant FA [29]. Therefore, Δ9-desaturase activity is critical for triglyceride storage in adipose tissue. While several pathways are involved in regulating FA composition, FAs produced from the precursors acetate and NADH, from the hydrolysis of triacylglycerols, and produced and deposited as rumen biohydrogenation metabolites can act as substrates for Δ9-desaturase. Adipose tissue develops in inter- and intra-muscular depots and both have a major impact on the quality and palatability of commercial beef. There is evidence for differential gene expression profiles in these two fat depots [30]. In this regard, the present study aimed to evaluate the regulation of *SCD* and *SREBP1*, genes strongly affecting the FA composition of subcutaneous adipose tissue, in three genetically diverse bovine breeds commercialized in the Basque region of northern Spain; Pirenaica, Salers and Holstein-Friesian [5, 31, 32]. Expression of *SCD1* did not differ significantly between Pirenaica bulls and heifers or among young cattle of Salers and Pirenaica. This may be partially explained by a similar feeding regimen, typically including concentrates, when meat production is the final purpose (Fig. 1). However, the content of Δ9 products, such as *cis*-MUFA, was higher in Salers bulls and Pirenaica heifers than corresponding bulls (Table 2). The Salers bulls and Pirenaica heifers, together with Holstein-Friesian cows, showed stronger correlations between *SCD1* and *SREBP*1 compared to Pirenaica bulls (Fig. 2). This suggests that, in Pirenaica breed, the FA composition is affected by the lipogenic gene regulation in a sex-dependent manner. Similarly, in a crossbred study, heifers exhibited higher *SCD1* mRNA levels and higher MUFA content than bulls

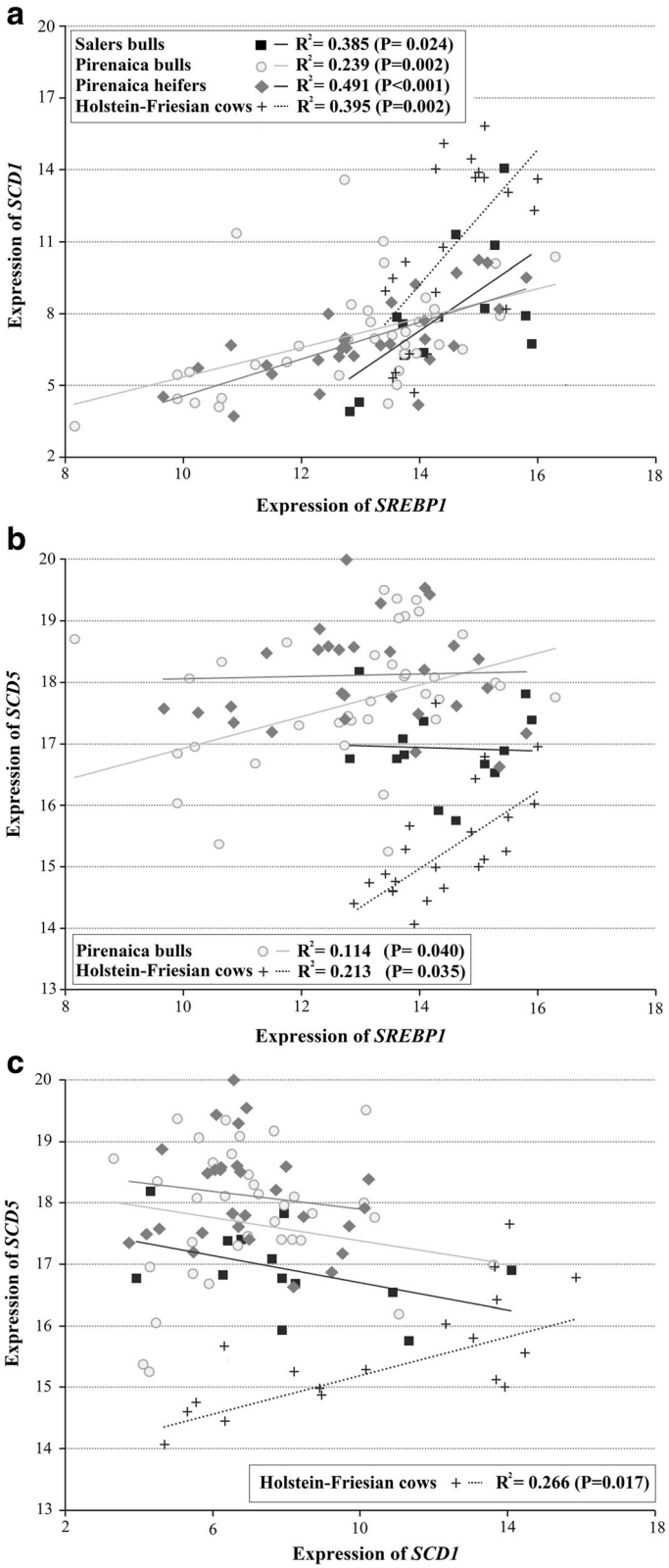

Fig. 2 Estimated linear regression equations between (**a**) *SCD1* and *SREBP1*, (**b**) *SCD5* and *SREBP1*, and (**c**) *SCD5* and *SCD1*

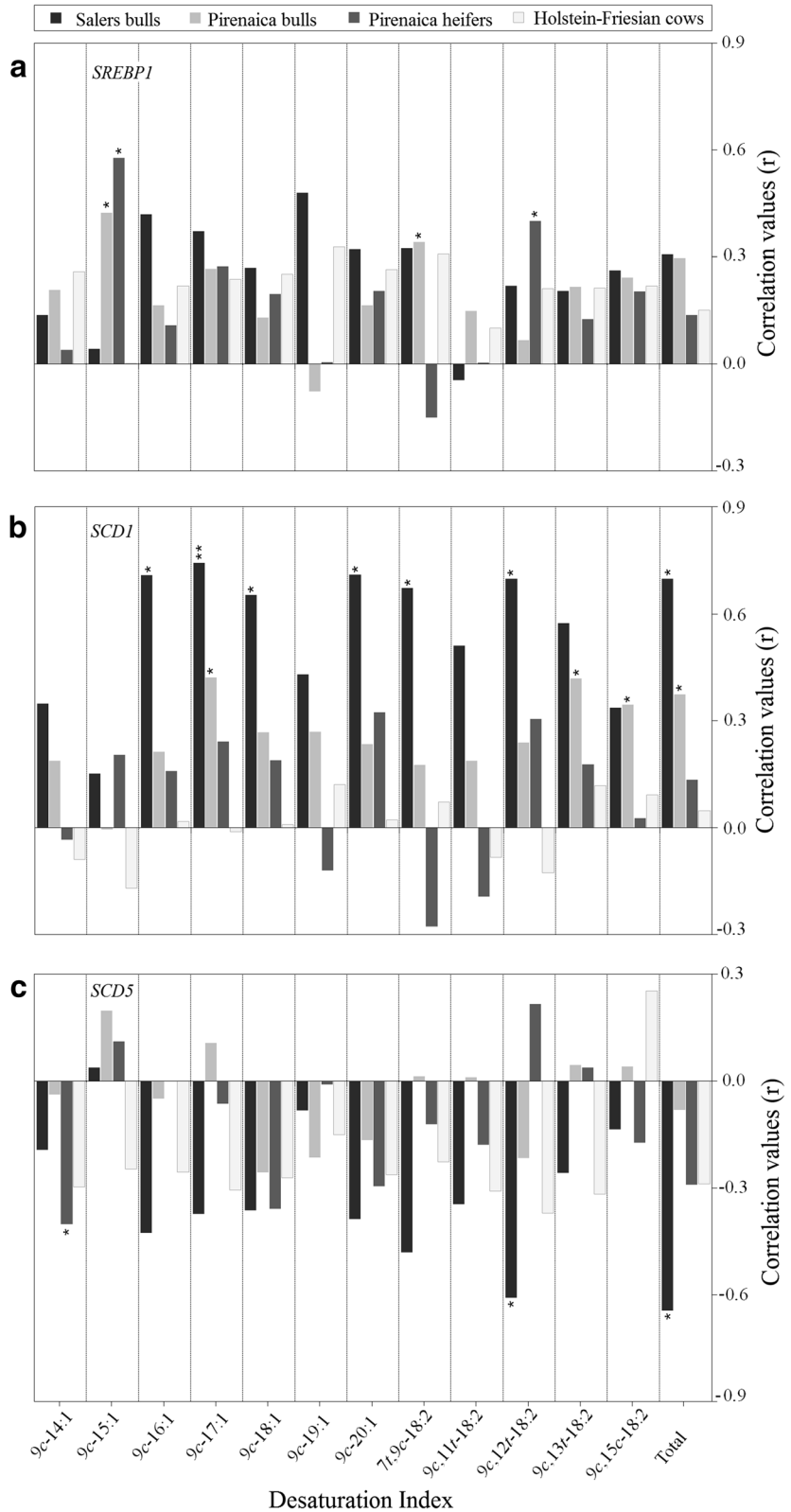

Fig. 3 Partial correlations controlling for age and HCW between gene expression of *SREBP1* (**a**), *SCD1* (**b**), *SCD5* (**c**) and desaturation indexes calculated from fatty acid composition data of cattle commercial types. $^{*}P < 0.05$, $^{**}P < 0.01$. Total is sum of all individual DIs. Desaturation indexes were calculated as [SCD product]/([SCD substrate] + [SCD product])

in subcutaneous adipose tissue [33], and a possible effect of sex hormones on enzymatic systems affecting lipid metabolism has been suggested [34]. Indeed, the growth hormone, sexually differentiated in mammals, seems to increase *SREBP*1 and *SCD1* gene expression in females [35]. Alternatively, age and diet have been demonstrated to influence adipocyte development in Pirenaica bulls [36]. Hence, the activation of *SCD1* due to a potentially higher concentrate consumption [19, 37] agrees with the greater total MUFA content of Salers and Pirenaica, while higher MUFA content in Pirenaica heifers than bulls seems to be more sex-dependent (Table 2). The greater variability in *SCD1* expression within Holstein-Friesian cows compared to young Pirenaica and Salers (Fig. 1) could be related to the less homogeneous diet and older age of these animals. Nevertheless, the generally lower *SCD1* expression observed in Holstein-Friesian cows was also reported in other mature culled cows [4], in which linoleic acid (18:2n-6) was suggested as the primary agent depressing *SCD* gene expression in adipose tissue [38].

We detected variability in *SCD5* mRNA expression levels among breeds ($P < 0.01$) and generally greater expression of *SCD1* relative to *SCD5* in all breeds. Lengi and Corl (2007) [11] also reported over 40-fold greater expression of *SCD1* compared to *SCD5* in adipose tissue of bulls (albeit with unspecified feeding). Variation among breeds was observed in the expression of both *SCD* isoforms, especially between beef and dairy cattle breeds (Salers and Pirenaica vs. Holstein-Friesian), suggesting that even if FA differences are generally small, there may still be differences in the underlying lipogenic gene expression or enzyme profile [39].

These differences in *SCD1* and *SCD5* expression levels (Fig. 1) also suggest that *SCD5* expression is more breed dependent than *SCD1* expression. However, it is also possible that *SCD5* expression is more sensitive than *SCD1* expression to other environmental factors (i.e., feeding) that differ among commercial types. Our results also revealed a potential opposite association between *SCD* isoforms within each breed. In general, this opposite correlation of DIs with *SCD*5 and *SCD*1 expression levels suggests that regulatory factors that upregulate *SCD*1 also downregulate *SCD*5 (and vice versa). However, since both *SCD* isoforms are expressed in adipose tissue, both may contribute to the maintenance of desaturation. In contrast to Pirenaica and Salers, this opposite association between *SCD1* and *SCD5* was not observed in Holstein-Friesian cows, a breed selected intensively for dairy production and most often utilized for beef production as cull cows at no specific age. This opposite pattern was more evident when the Holstein-Friesian sample was stratified by age (data not shown). Furthermore, Holstein-Friesian cows may exhibit side effects of dairy selection that differentially affect the genetic sequences containing *SCD* genes, thereby influencing transcriptional regulation. Moreover, variability in the regulatory DNA sequences of *SCD* genes may confer differences in gene expression and physiological changes that could also explain the different correlation patterns with DIs observed among commercial types. 9*c*-14:1 DI has been reported as the best indicator of overall SCD enzyme activity by Corl et al. (2002) [40]. Feedstuffs are normally devoid of 9*c*-14:1 and, therefore, this FA is produced by de novo FA synthesis. In mammary gland, significant correlation between 9*c*-14:1 DI and *SCD1* expression was observed, whereas 9*c*-14:1 DI and *SCD5* correlation was not [41]. We did not observe significant correlation between 9*c*-14:1 DI and *SCD1*, similarly to a previous study in intestinal adipose tissue, skeletal muscle or mammary gland [42]. However, we observed that 9*c*-14:1 DI and *SCD5* were negatively correlated in subcutaneous adipose tissue of Pirenaica heifers ($P < 0.05$). Thus, these results suggest that *SCD* gene expression may directly affect 9*c*-14:1 content, but 9*c*-14:1 DI correlation with *SCD5* and *SCD1* might be breed and tissue specific as well.

As previously reported by Horton et al. [14], *SCD1* and *SREBP1* appear to be directly related as there was a significant linear association between these two genes in all commercial types studied (Fig. 2a). Differences in slope and coefficient of determination (R^2) values, however, revealed variability in this relationship among commercial types. A previous study suggested that the FA synthesis pathway is regulated in a coordinated manner by the SREBP family of membrane-bound transcription factors, and regulation of *SCD1* by *SREBP1* via the SRE binding site of *SCD1* has been demonstrated [43].

Significant correlation between *SCD5* and *SREBP1* specifically observed in Pirenaica bulls and Holstein-Friesian cows suggest that *SCD5* expression may be more variable among commercial types than *SCD1* expression, possibly due to differences in regulation by *SREBP1*. For example, according to Lengi and Corl (2012) [16], the early growth response protein 2 (*EGR2*) and *SREBP1* may bind to the same DNA site of the bovine *SCD5* promoter. They observed that expression of *EGR2* or *SREBP1* did not increase endogenous *SCD5* mRNA expression but did activate a truncated bovine *SCD5* promoter luciferase reporter constructs in human JEG3 cells. Therefore, they attributed the lack of increase in *SCD5* expression to the presence of additional negative-regulation sites in this gene. In our case, the absence of significant differences in other commercial types could be due to breed or other environmental factors that could, in part, modulate these putative negative-regulation sites.

Among these commercial types of the Basque region, correlations between lipogenic genes (*SCD1* and *SREBP1*)

and calculated DIs were stronger in Salers bulls than Pirenaica bulls and heifers (Fig. 3). In Holstein-Friesian cows, correlations were not significant (Fig. 3). However, *SREBP1* and *SCD1* correlations with DIs became significant ($P < 0.05$) when computed without age and HCW as covariates (data not shown). Moreover, correlations between *SCD1* expression and DIs were slightly higher in younger Holstein-Friesians, while *SCD5* correlations with DIs were higher in older Holstein-Friesians. This suggests an effect of age on gene expression–DI correlations in Holstein-Friesian cows. In addition to 16:1 and 18:1 [42], positive correlations between *SCD1* and calculated DIs were observed for other FA species, suggesting desaturase activity also targets minor FAs of subcutaneous fat. In this regard, DIs and MUFA content were more susceptible to the expression of lipogenic genes in Pirenaica heifers than bulls. Furthermore, the effect of lipogenic gene expression on DIs was stronger in Salers than Pirenaica heifers. Our findings are supported by a previous study [13] suggesting that the FA composition of subcutaneous adipose tissue is mainly dependent on genetic background, which may in turn indicate inter-breed differences in lipid metabolism. The effect of breed appears to be more strongly associated with *SREBP*1 expression level than *SCD1* or *SCD5* expression level (Fig. 1), whereas the underlying regulation of *SCD1* and *SCD5* could be responsible for inter-breed differences in DIs and FA profiles.

We also report an opposite effect of *SCD* isoforms on certain DI values, especially in Salers bulls. This stronger pattern may stem for a more homogeneous production system (Salers breeder, personal communication) that may reduce the influences of extraneous factors. Positive correlations between DIs and *SCD*1 (Fig. 2b) and contrasting negative correlations between DIs and *SCD*5 (Fig. 2c) are likely due to genetic compensation. Lower expression of one *SCD* isoform could well be compensated for by upregulation of the other isoform (Fig. 1). This compensation theory was previously suggested in *Caenorhabditis elegans* [44]. The reciprocal expression observed between different isoforms and the underlying epigenetic processes require further investigation.

The CLA isomer 10 *t*,12*c*-18:2 was examined because it was previously described as an important inhibitor of *SCD1* in dairy cattle [25]. In our study, although Pirenaica heifers accumulated the highest amounts of 10 *t*,12*c*-18:2 in subcutaneous adipose tissue (Table 2), no significant correlation was observed between 10 *t*,12*c*-18:2 and lipogenic gene expression (data not shown). Nevertheless, both isoforms may be differently regulated. In contrast to *SCD1*, which tends to be reduced by 10 *t*,12*c*-18:2 [45], *SCD5* appears to be more stable due to lack of an N-terminal PEST sequence for degradation [11]. However, further research is needed to establish relationships among DIs and SCD isoform mRNA expression levels, and to clarify the effects of 10 *t*,12*c*-18:2 on bovine adipose and muscle tissues. Analysis of lipogenic gene expression changes with dietary treatment in ruminant species as well as promoter sequencing would provide valuable insight into the regulation of these genes and their impact on the synthesis of MUFAs and PUFAs.

Conclusion

The present study suggests that the differences in subcutaneous fat FA composition among bovine commercial types of the Basque region are related to genetic variability in lipogenic gene expression. The expression of lipogenic genes in Salers bulls showed clear effects on desaturation indexes and FA composition. All breeds show a strong correlation between *SREBP1* and *SCD1* expression. In addition, distinct correlations between *SCD* isoforms and DIs suggest a novel genetic compensation mechanism between *SCD1* and *SCD5* that warrants further investigation.

Abbreviations

c: *Cis*; CLA: Conjugated linoleic acid; DI: Desaturation index; FA: Fatty acid; HCW: Hot carcass weight; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; SCD: Stearoyl-CoA desaturase; SFA: Saturated fatty acids; SREBP: Sterol regulatory element-binding protein; *t*: *Trans*; UFA: Unsaturated fatty acids

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding authors on reasonable request.

Authors' contributions

DG analyzed the overall experimental data used in this study and wrote the manuscript. NA analyzed and interpreted fatty acid composition in adipose tissue of cattle. AA and LJRB performed statistical analyses. MT analyzed and interpreted lipogenic gene expression data. ALO and MMP participated in discussion. All authors contributed to drafting the manuscript and gave final approval of the version to be published.

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Masaaki Taniguchi: Molecular genetics analyses on meat quality of beef and pork using high throughput technologies such as single nucleotide polymorphisms (SNP) array and gene expression microarray.
Noelia Aldai: Lipids in animal science: factors influencing the composition, strategies to improve the nutritional quality of meat, and comprehensive analytical methods that demonstrate their value.

Ethics approval
All animals were handled at slaughterhouse following the European Council Regulation (EC) N° 1099/2009. All material was sampled after slaughter and therefore do not require any ethical consideration or approval.

Competing interests
The authors declare that they have no competing interests.

Publisher's Note

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APPENDIX V.

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ORIGINAL ARTICLE

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Effect of a genetic polymorphism in SREBP1 on fatty acid composition and related gene expression in subcutaneous fat tissue of beef cattle breeds

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Abstract

Sterol regulatory element-binding factor 1 (*SREBP1*) plays an important role in the lipogenesis which affects fatty acid (FA) composition in backfat and consequently influences beef nutritional quality. This study analyzed the association of 84 bp-indel, both short (S) and long (L) alleles in intron 5 of *SREBP1*, with FA composition and gene expression of *SREBP1* in backfat of northern Spanish beef breeds (Pirenaica, Salers and Holstein-Friesian). Phylogenetic analysis suggests that 84 bp-indel of ruminants is a highly conserved region compared with those in the full-length sequence of intron 5 or mRNA of *SREBP1* among species. Overall, higher content of polyunsaturated FAs was observed in SL genotype compared to LL genotype of 84 bp-Indel ($p < .05$). In particular, in Pirenaica, SL genotype was associated with a higher content of stearic (18:0), α-linolenic (18:3n-3) acid, and total n-3 content ($p < .05$). However, the gene expression of *SREBP1* did not differ among genotypes of 84 bp-Indel ($p > .05$).

KEYWORDS

beef, fatty acid composition, gene expression, genetic polymorphism, meat quality

1 | INTRODUCTION

It is widely recognized the beneficial effects of unsaturated fatty acids (UFA) in human health, which has been also emphasized by the World Health Organization (Burlingame et al., 2009; Vannice & Rasmussen, 2014). Thus, enhancing the UFA profile in animal-derived products improves the nutritional quality of them (Brooks et al., 2011). In terms of polyunsaturated fatty acids (PUFA), n-3, and n-6 are the most common, being linoleic (18:2n-6) and α-linolenic (18:3n-3) acids the most abundant. However, humans are unable to synthesize them and, therefore, they are considered essential dietary fatty acids (FA). Lipogenesis is nutritionally controlled at the transcriptional level by a common family of transcription factors designated sterol regulatory element binding proteins (SREBPs; Brown & Goldstein, 1997). These transcription factors have an important role in controlling around 30 genes affecting the synthesis of FAs, triacylglycerols, and glycerophospholipids (Shimomura et al., 1997; Zimin et al., 2009), and are considered also master regulators of cholesterogenesis (Eberlé et al., 2004).

The three major SREBP isoforms named *SREBP1a*, *SREBP1c*, and *SREBP2* are identified in humans, rodents, and also in cattle (Horton et al., 2002). Over time, SREBPs have been conserved from fungi to mammals, and the phylogenetic analysis demonstrated the existence of predicted SREBP homologs across Eukarya (Osborne & Espenshade, 2009). Moreover regulation and functions of these transcription factors and their implication in several pathologies (Bao et al., 2016; Picard et al., 2018) and also their association with nutritionally healthy FA profiles in milk and meat (Bauman et al., 2011; Ladeira et al., 2016) are of permanent interest.

SREBPs regulate gene transcription activation by binding to the sterol regulatory element (SRE) sequences present in the promoter of downstream genes, including stearoyl-CoA desaturase (*SCD*) genes (Shimano, 2001). Therefore, differences in expression level and polymorphisms of *SREBP* gene may affect the expression of other downstream genes, leading to differences in FA composition of adipose tissues. In this regard, several studies have reported the association of genetic polymorphisms within *SREBP* genes with FA composition in cattle (Hoashi et al., 2007; Rincon et al., 2012), whereas mRNA expression of *SREBP1* was recently correlated with differences in FA profile of several bovine breeds (Gamarra et al., 2018).

Hoashi et al. (2007) identified an interesting 84 bp insertion/deletion polymorphism (84 bp-indel; rs133958066) of intron 5 of *SREBP1*, which was associated with higher monounsaturated FA (MUFA) content and lower melting point of intramuscular fat. This polymorphism has been widely investigated in cattle breeds such as Holstein-Friesian, Hereford and Angus, as well as in *Bos indicus* and other local breeds (Kaneda et al., 2011; Öztabak et al., 2013; Proskura, 2013). The 84 bp-indel has also been associated with growth traits (Huang et al., 2011) and milk production (Proskura et al., 2017), while some other studies have investigated its association with FA composition of backfat and muscle (Barton et al., 2010; Han et al., 2013; Lee et al., 2013; Matsuhashi et al., 2011; Xu et al., 2013). However, there are no studies investigating the presence/absence of 84 bp-indel along with *SREBP1* gene expression and their association with the FA profile. In this study, we firstly investigated a sequence similarity of the coding and conserved non-coding regions of *SREBP1* among ruminants. Second, we aimed to analyze the association between the FA profile in backfat and the 84 bp-indel of *SREBP1* gene in several commercial northern Spanish beef cattle breeds (Pirenaica, Salers, and Holstein-Friesian). Furthermore, we discussed the possible effect of the 84 bp-indel on gene expression of *SREBP1* and the lipogenic genes regarding the FA composition.

2 | METHODS

2.1 | Sample collection

Bovine breeds typically destined to meat production in northern Spain (Basque region) were used. Samples groups were organized in four commercial types according to breed, sex, and age of slaughter (Salers bulls, Pirenaica bulls, Pirenaica heifers and Holstein-Friesian cows). Description of commercial types and sample collection details were previously published (Aurtenetxe et al., 2017; Gamarra et al., 2018). Briefly, Salers and Pirenaica calves were of similar age (average of 12.9 ± 1.4 months), while Holstein-Friesian cows were slaughtered at 70.0 ± 19.4 months (cull cows), which are common commercial types in the region. Sample collection was designed according to the Bovine Identification Document data and inferred relationships (parentage and sibships) computed from 29 microsatellites (Software Colony 2.0. 6.2). In this regard, neither parentage nor maternal half-sibs were observed, and paternal half-sibs were maintained at low frequencies (Pirenaica, 0.009; Salers 0.013; Holstein-Friesian, 0.019).

Subcutaneous adipose tissue samples from the 5-6th rib and muscle samples from the neck (*sternomandibularis*) were collected from the left half carcasses (13 Salers bulls, 37 Pirenaica bulls, 28 Pirenaica heifers, and 21 Holstein-Friesian cows; $n = 99$). Both, backfat for FA analysis and neck muscle samples for genotyping, were stored in plastic bags with the air removed. Backfat samples for RNA analysis were, however, preserved in RNAlater™ (Ambion). All samples were transported to the laboratory in insulated coolers and stored immediately at −80°C.

2.2 | Analysis of fatty acid composition

The FA analysis was previously described in Gamarra et al. (2018). Briefly, a 50 mg sample of backfat was freeze-dried and directly derivatized with sodium methoxide (Kramer et al., 1997) and for quantitation purposes internal standard (23:0 methyl ester) was added prior to derivatization. Resulting FA methyl esters (FAME) were analyzed by gas chromatography with flame ionization detection using two complementary 100 m columns; SP-2560 (Kramer et al., 2008) and SLB-IL111 (Delmonte et al., 2012). FA data were reported in mg/g of fat.

2.3 | Genotyping of bovine SREBP1

DNA was extracted from 5 mg of neck muscle tissue with Proteinase-k and single lysis-salting out method. DNA samples were quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) and diluted to 50 ng/μl. First, *SREPB1* 84 bp-indel was detected by simple sequence length polymorphism (SSLP) of PCR amplicons. The PCR amplifications for *SREBP1* were carried out in a final volume of 25 μl containing 0.5 U of KOD-plus DNA polymerase (TOYOBO), 2.5 μl of 10X PCR buffer, 1 mM $MgSO_4$, 25–50 ng of genomic DNA, 0.2 mM dNTP (Takara Biotechnology Co, Ltd, Shiga, Japan), and 0.4 μM of each primer. Primers used were F: 5′-TGACCTCCCAGATACAGCAG-3′ and R: 5′-GCTACTCAACAGCAGGATCTC-3′. Amplification conditions were 94°C for 2 min, 30 cycles of 94°C for 5 s, 60°C for 15 s, 68°C for 1.5 min, and a final extension at 72°C for 10 min. Amplification products were subjected to electrophoresis on

1.5% agarose gels and visualized with GelRed (Biotium Inc.) and UV light. The individuals were genotyped according to the length of PCR products. Then, Real-time PCR and high resolution melting (HRM) were optimized to genotype the 84 bp-indel polymorphism. CFX-96 Real-Time System (Bio-Rad Laboratories) and following primers (5′-AGAAACGCTACCGCTCTTCC-3′ and 5′-GCTACTCAACAGCAGGATCTC-3′), 0.75 μM of each universal primer, 2.5 μl of SsoFast^{TM} EvaGreen® supermix and 1 μl of DNA template (1 ng/μl) were used. PCR and HRM were performed following an initial denaturation at 98°C for 2 min; 40 cycles at 98°C for 5 s, and 50°C for 30 s (Lopez-Oceja et al., 2017). Plate reads were taken after the initial step of 95°C for 30 s and 60°C for 2 min, and the melting curve went from 65°C to 95°C with an increment of 0.5°C every 5 s.

2.4 | Gene expression analysis of bovine lipogenic genes

Total RNA was extracted using Rneasy Lipid Tissue kit (Qiagen Inc.) and analyzed with Custom TaqMan Assay (Applied Biosystems) to measure the relative expression level of *SREBP1* and *SCD* isoforms (*SCD1* and *SCD5*) following the conditions and details reported in Gamarra et al. (2018). Reverse transcription was performed using ReverTra Ace reverse transcriptase (TOYOBO), while Real time PCR was conducted to measure the relative expression levels of each candidate gene in multiplex with an internal control (18S rRNA Endogenous Control VIC/TAMRA Probe, Primer Limited) by the co-application of reverse transcription method (Co-RT). Real-time PCR was performed in triplicate using the ABI Prism 7500 Sequence Detection System (Applied Biosystems) and PCR efficiency was calculated per sample and amplicons using LinRegPCR (Heart Failure Research Center). Efficiencies were over 90% for all samples and correlation coefficients were over 0.99. The comparative threshold cycle method (ΔCt) was employed to calculate the relative gene expression based on this formula: $\Delta Ct = (Ct_{\text{target gene}} - Ct_{\text{18S rRNA gene}})$.

2.5 | Statistical analysis

For the phylogenetic analysis, alignments of *SREBP1* mRNA sequences among several ruminants (cattle, zebu, buffalo, sheep, goat) and other mammals (horse, pig, dog, mouse, rat, chimpanzee, human) were made with MUSCLE algorithm (Edgar, 2004). The data used for sequence alignments were obtained from NCBI's GenBank (Benson et al., 2018; accession numbers reported in Table S1). Phylogenetic tree inferred from the concatenated alignment of *SREBP1* mRNA sequences was constructed using the maximum likelihood method (100 bootstrap) in Unipro UGENE v31 (Okonechnikov et al., 2012). Alignments of the 84 bp-indel from bovine *SREBP1* and homologous introns of aforementioned mammals were also computed. Similarity percentages were calculated based on nucleotide similarity among sequences.

To study *SREBP1* genotype and FA associations, IBM SPSS Statistics 22 was used (SPSS Inc., IBM Corporation). First of all, data were checked for normality and homoscedasticity. Then, the following general linear model was used for analysis of variance (ANOVA): $Y_{ijk} = \mu + IP_i + GE_j + e_{ijk}$ where Y_{ijk} = dependent variables (FAMEs); μ = mean value; IP_i = effect of 84 bp-indel polymorphism of *SREBP1* (SS: Homozygote, small type with 84 bp deletion; LL: Homozygote, long type with 84 bp insertion; SL: Heterozygote with 84 bp deletion and insertion); GE_j = covariate, gene expression of *SREBP1* (ΔCt); e_{ijk} = random error component. Gene expression of *SREBP1* (*GE*) was included in the statistical model as a covariate. The fixed effects of age at slaughter, hot carcass weight, fat cover, and effect of sire were checked but not included in the model ($p > .05$). The additive model (SS, SL, and LL) and the recessive model (SS/SL and LL) were evaluated. Least significant difference (LSD) post hoc test was applied for multiple comparison of means among genotypes studied considering 84 bp-indel frequencies.

Linear regression analyses were conducted to examine the associations among the genotype of 84 bp-indel and individual FAs following a linear regression model:

$$Y_i = \beta_0 + \beta_1 X_i + e_i,$$

where y_i is dependent variable (FAs); β_0 is overall intercept; β_1 is slope coefficient; X_{i1} is genotype of 84 bp-indel polymorphism of *SREBP1* (SS/SL or LL); e_i is random error component.

Regression and correlation analyses between *SREBP1* gene expression and FA composition were conducted in R (R Core Team, 2019) and figures were produced using the packages ggplot2 and ggally (Schloerke et al., 2020; Wickham, 2016). Pearson correlations were computed among gene expression (ΔCt) of *SREBP1*, considering the 84 bp-indel, and main individual and FA groups to evaluate differences among commercial types. Partial Pearson correlations corrected by breed and sex were computed. Correlations adjusted for GE were also computed, whenever it was permitted by sample size, among gene expression of downstream genes (*SCD1* and *SCD5*) and main individual and FA groups considering the 84 bp-indel genotypes, since it represented more accurately the effects of *SREBP1*-indel on *SCD1* and *SCD 5* genes and FAs. Significance was declared at $p \leq .05$.

3 | RESULTS

3.1 | Evaluation of SREBP1 genotyping data

Detecting the SSLP of bovine *SREBP1*, two alleles (S, 698 bp band; L, 782 bp band) and three genotypes (SS, SL, and LL) were identified in cattle commercialized in the Basque region (Figure 1a). Then, melting profiles of each genotype were grouped into distinct clusters which were assigned as reference clusters (Figure 1b). All samples from the same genotype were grouped in the same cluster with confidence levels over 98%; melting temperatures

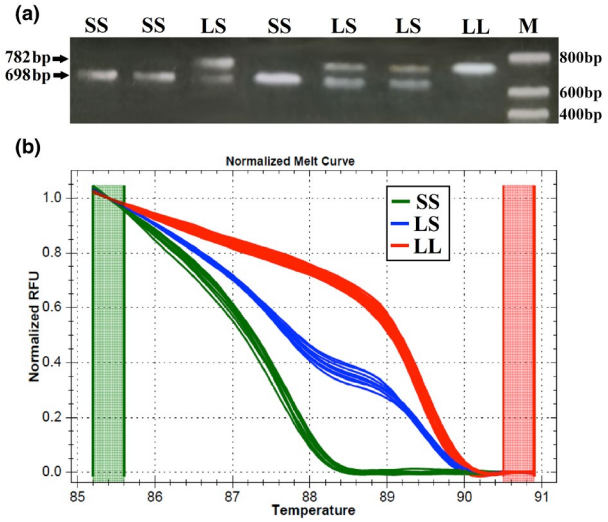

FIGURE 1 Genotyping of bovine *SREBP1* gene. (a) Electrophoretic patterns of the DNA region containing the indel (1.5% agarose gel) and (b) normalized melting curves showing different 84bp-indel genotypes (HRM) of bovine *SREBP1* gene. LL, Long homozygote; LS, Long-Small heterozygote; M, marker; SS, Small homozygote

were stable and low standard deviations for each genotype were observed.

3.2 | SREBP1 alignment among mammals

The bovine sequence of *SREBP1* mRNA demonstrated high similarity with the sequence of other ruminants such as zebu (*Bos indicus*, 99%), buffalo (*Bubalus bubalis*, 85%), sheep (*Ovis aries*, 93%), and goat (*Capra hircus*, 96%; Figure 2). Likewise, it showed a moderate to high similarity when compared with other mammals such as pig (*Sus scrofa*, 82%), dog (*Canis familiaris*, 82%), horse (*Equus caballus*, 73%), mouse (*Mus musculus*, 72%), and rat (*Rattus norvegicus*, 73%). Furthermore, bovine *SREBP1* mRNA sequence showed higher similarity to *SREBP1c* isoform of hominids, such as human (*Homo sapiens*) or chimpanzee (*Pan troglodytes*; 82%; Figure 2a), than to *SREBP1a* (65%) of the same hominids. In general, high bootstraps (of 100) were observed in the tree nodes among ruminants, but also pig, demonstrating the strength of these alignments. Node bootstraps were lower for horse, dog, and hominids since these

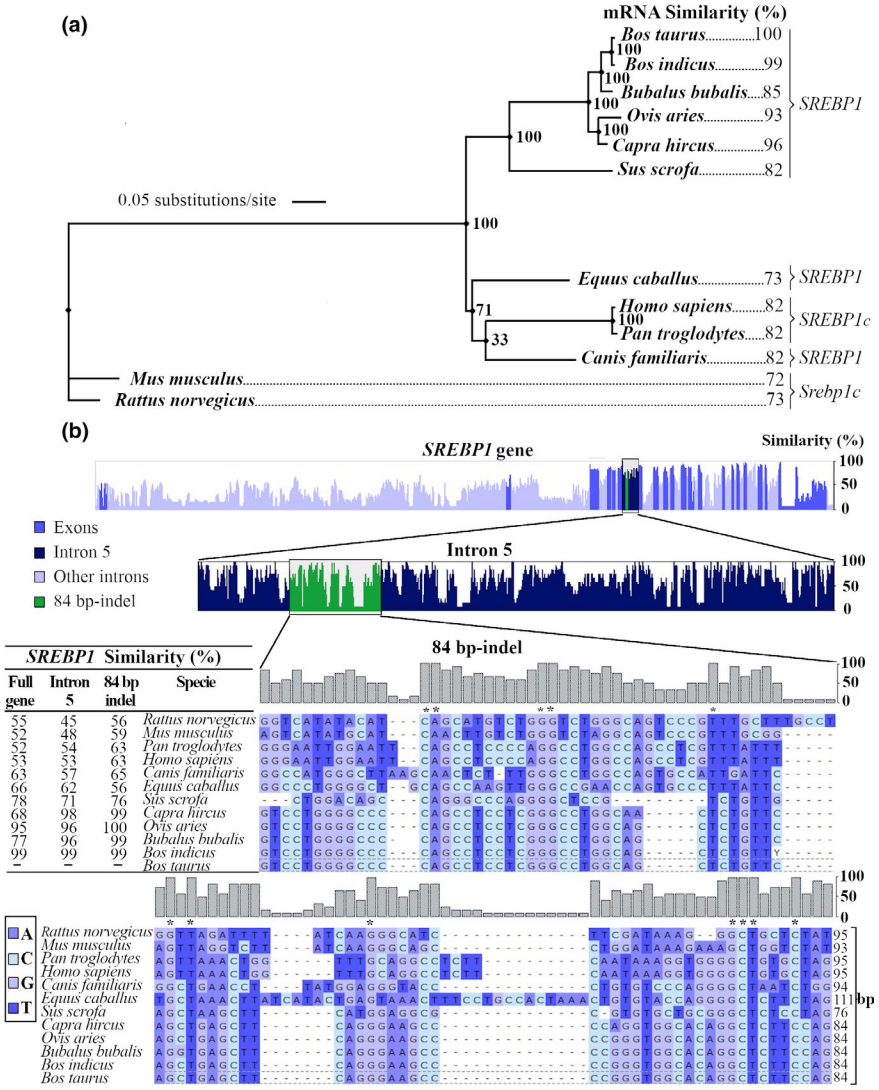

FIGURE 2 Representation of the nucleotide sequence similarities of *SREBP1* gene among mammalians. (a) maximum-likelihood tree using mRNA of *SREBP1* in different mammals (the length of branches represents the expected number of substitutions per site) and the percentage of bootstrap replicates (up to 100) that support each node. and the (b) area graphs (histograms) showing the similarity and the alignment of bovine genomic sequence of *SREBP1*, intron 5 and 84 bp-indel region along with homologous sequences of different mammals

TABLE 1 Effect of genotype (SS/SL vs. LL) on the fatty acid content (mg/g fat) of subcutaneous adipose tissue of bovine commercial types (Salers bulls, Pirenaica bulls, and heifers)

	Salers bulls			Pirenaica bulls			Pirenaica heifers			Holstein-Friesian cows
Fatty acid	SS/SL ($n = 7$)	LL ($n = 6$)	*p*-value	SS/SL ($n = 9$)	LL ($n = 28$)	*p*-value	SS/SL ($n = 9$)	LL ($n = 19$)	*p*-value	LL ($n = 21$)
SFA	409 ± 23.7	365 ± 25.6	NS	388 ± 23.4	354 ± 13.3	NS	369 ± 12.9	377 ± 8.8	NS	406 ± 83.5
16:0	231 ± 9.8	208 ± 10.6	NS	216 ± 12.6	204 ± 7.10	NS	213 ± 8.1	228 ± 5.5	NS	228 ± 52.8
18:0	128 ± 12.11	117 ± 13.08	NS	131 ± 9.67	107 ± 5.48	.037	111 ± 5.27	104 ± 3.61	NS	135 ± 60.25
MUFA	411 ± 12.1	414 ± 13.0	NS	373 ± 19.3	359 ± 11	NS	438 ± 11.3	439 ± 7.80	NS	356 ± 110
9*c*-16:1	32.1 ± 2.25	33.4 ± 2.43	NS	26.9 ± 2.68	29.9 ± 1.52	NS	32.7 ± 2.67	36.4 ± 1.83	NS	34.4 ± 24.47
9*c*-18:1	300 ± 8.84	304 ± 9.55	NS	269 ± 14.29	247 ± 8.1	NS	300 ± 11.83	303 ± 8.12	NS	261 ± 72.15
PUFA	30.2 ± 2.41	32 ± 2.61	NS	27.7 ± 2.50	24.5 ± 1.42	NS	24.4 ± 1.88	23.3 ± 1.29	NS	14.1 ± 4.21
n-6	28.0 ± 2.30	29.7 ± 2.49	NS	25.2 ± 2.37	22.4 ± 1.35	NS	22.1 ± 1.77	21.1 ± 1.22	NS	11.9 ± 3.89
18:2n-6	26.5 ± 2.29	28.3 ± 2.47	NS	24 ± 2.37	21.2 ± 1.35	NS	20.2 ± 1.72	19.4 ± 1.18	NS	10.6 ± 3.76
18:3n-6	0.102 ± 0.012	0.11 ± 0.013	NS	0.085 ± 0.018	0.13 ± 0.010	0.037	0.156 ± 0.018	0.165 ± 0.012	NS	0.141 ± 0.053
n-3	2.11 ± 0.134	2.17 ± 0.145	NS	2.47 ± 0.206	1.97 ± 0.117	0.042	2.23 ± 0.169	2.02 ± 0.116	NS	2.08 ± 0.623
18:3n-3	1.87 ± 0.133	1.9 ± 0.144	NS	2.21 ± 0.197	1.72 ± 0.111	0.040	1.91 ± 0.156	1.67 ± 0.107	NS	1.7 ± 0.583

Note: The unit of fatty acid is denoted with mg/g of fat tissue. Values represented in this table are least square means ± standard deviations.

The FA group descriptions was referred to Gamarra et al. (2018).

mammals phylogenetically belong to different animal families. *SREBP1* full gene alignment showed higher similarity among mammals in exon regions, and interestingly, the intron 5 showed higher similarity than other introns (Figure 2b). In ruminants, full *SREBP1* gene alignment showed the lowest similarity in buffalo and goat (<80%). The alignment of intron 5 showed that similarity is higher in ruminants (99%–96%) compared with non-ruminant mammals (71%–45%; Figure 2b). Interestingly, alignment of 84 bp-indel region showed the highest similarity among ruminants (≥99%), and similarity was lower in other mammals (76%–56%). In general, the 84 bp-indel region is highly conserved among mammals even this polymorphism is located in a non-coding region. However, S allele was not observed in any of the intron sequences reported in GenBank for the compared species. Larger gaps were due to sequence differences among mammals, for example as observed in horse (*Equus caballus*).

3.3 | Allele frequencies and associations with the fatty acid composition

The frequency of the minor allele (S) was higher in Salers bulls (0.385) compared to Pirenaica heifers (0.214) and bulls (0.135). In contrast, Holstein-Friesian cows did not show 84 bp deletion (S allele) and all samples presented the LL genotype. When association analyses were performed (Table 1), overall, few FA differences were

TABLE 2 Effect of genotype (SS vs. SL vs. LL) on the fatty acid content (mg/g fat) of subcutaneous adipose tissue of all bovine commercial types[a] (Salers bulls, Pirenaica bulls, and Pirenaica heifers) and Pirenaica breed[b] (bulls and heifers)

	All group ($n = 78$)[a]				Pirenaica breed ($n = 65$)[b]			
	SS ($n = 7$)	SL ($n = 18$)	LL ($n = 53$)	P^*	SS ($n = 4$)	SL ($n = 14$)	LL ($n = 47$)	P^*
SFA	363 ± 22.36	397 ± 13.9	363 ± 8.1	NS	334 ± 29.6	392 ± 15.6	363 ± 8.52	NS
16:0	208 ± 12.12	223 ± 7.53	213 ± 4.39	NS	190 ± 16.5	221 ± 8.71	214 ± 4.76	NS
18:0	112.3 ± 9.5[a]	127 ± 5.91[a]	106 ± 3.44[b]	.012	107 ± 12.2[a]	126 ± 6.42[a]	105 ± 3.51[b]	.022
MUFA	416 ± 18.06	402 ± 11.23	395 ± 6.54	NS	405 ± 24.6	399 ± 12.9	393 ± 7.09	NS
9c-16:1	32 ± 2.9	30 ± 1.81	33 ± 1.05	NS	30.2 ± 3.99	29.1 ± 2.11	32.7 ± 1.15	NS
9c-18:1	300.9 ± 15.01	282.7 ± 9.33	274.3 ± 5.44	NS	290 ± 20.1	278 ± 10.6	271 ± 5.79	NS
PUFA	26.36 ± 2.68	27.97 ± 1.66	24.77 ± 0.97	NS	23.7 ± 3.44	27.2 ± 1.82	23.9 ± 0.99	NS
n-6	24.16 ± 2.55	25.49 ± 1.59	22.65 ± 0.93	NS	21.4 ± 3.267	24.7 ± 1.724	21.8 ± 0.942	NS
18:2n-6	22.7 ± 2.524	24 ± 1.569	21.2 ± 0.915	NS	20 ± 3.236	23.2 ± 1.707	20.3 ± 0.933	NS
18:3n-6	0.106 ± 0.019	0.117 ± 0.012	0.141 ± 0.007	NS.	0.108 ± 0.027	0.12 ± 0.014	0.145 ± 0.008	NS
n-3	2.09 ± 0.205[a]	2.37 ± 0.128[a]	2.01 ± 0.074[b]	.050	2.13 ± 0.288[a]	2.42 ± 0.152[a]	1.99 ± 0.083[b]	.047
18:3n-3	1.83 ± 0.195[a]	2.09 ± 0.121[a]	1.72 ± 0.071[b]	.034	1.84 ± 0.272[a]	2.15 ± 0.152[a]	1.69 ± 0.078[b]	.028

[a]Corrected by breed and sex.

[b]Corrected by sex.

*p indicates p-values of the analysis of variance. Significant difference is defined as $p < .05$, otherwise it is denoted as NS (not significant) or NS. (Trend, $p < .1$). The unit of fatty acid is denoted with mg/g of fat tissue; The values represented in this table are least square means ± standard deviations; The FA group descriptions (SFA, MUFA, and PUFA) was referred to Gamarra et al. (2018).

TABLE 3 Regression equations between the genotype (SS/SL vs. LL) and the fatty acid content (mg/g fat) of subcutaneous adipose tissue of Pirenaica (bulls & heifers), Pirenaica bulls and Pirenaica heifers

	Pirenaica bulls & heifers			Pirenaica bulls			Pirenaica heifers		
Fatty acid	Slope	*SEM*	*p*	Slope	*SEM*	*p*	Slope	*SEM*	*p*
18:0	−15.8	6.740	.022	−24.2	10.9	.034	−6.81	6.41	NS
n-6	−2.04	1.788	NS	−2.81	2.705	NS	−0.948	2.16	NS
18:2n-6	−1.96	1.784	NS	−2.87	2.702	NS	−0.849	2.10	NS
18:3n-6	0.023	0.016	NS	0.045	0.021	.036	0.008	0.022	NS
n-3	−0.371	0.156	.020	−0.504	0.234	.038	−0.202	0.206	NS
18:3n-3	−0.377	0.149	.015	−0.488	0.224	.036	−0.235	0.189	NS

Abbreviation: *SEM*, Standard error of the mean.

p indicates *p*-values of the analysis of variance. Significant difference is defined as $p < .05$, otherwise it is denoted as NS (not significant).

observed among genotypes within each of the bovine commercial types studied. In Pirenaica bulls, higher 18:0, 18:3n-3, and total n-3 contents were observed in SS/SL compared to LL genotype ($p < .05$), whereas only the minority 18:3n-6 content was higher in LL genotype. In the additive model (Table 2), 18:0, 18:3n-3, and total n-3 content were also higher in SL genotype compared to LL genotype in Pirenaica breed ($p < .05$). Linear regressions were conducted to relate the genotype of 84 bp-indel to FA contents. Pirenaica breed (bulls and heifers together) and Pirenaica bulls alone showed significant linear regressions for 18:0, 18:3n-3, and n-3 contents (Table 3).

SREBP1 gene expression was analyzed in order to elucidate any direct relationship of each genotype (84 bp-indel) and gene expression (Figure 3). Significant differences were not observed among genotypes for each breed, neither between Pirenaica bulls and heifers ($p > .05$; Figure 3). Only *SREBP1* gene expression of LL genotype has shown to be higher in Pirenaica breed than Salers and Holstein-Friesian ($p < .05$; Figure 3a), although *SCD1* and *SCD5* gene expression did not show significant differences among 84-bp indel genotype ($p > .05$; Figure S1). Only Holstein-Friesian demonstrated a significant correlation between *SREBP1* expression and 16:0, MUFA including 9*c*-16:1 and 9*c*-18:1 FA content ($p < .01$; Figure 4). Correlations among bovine gene expression and contents of FA separated by commercial types and 84 bp-indel were also performed. Overall, few significant correlations were observed among gene expression and individual FAs (Figures S1 and S2).

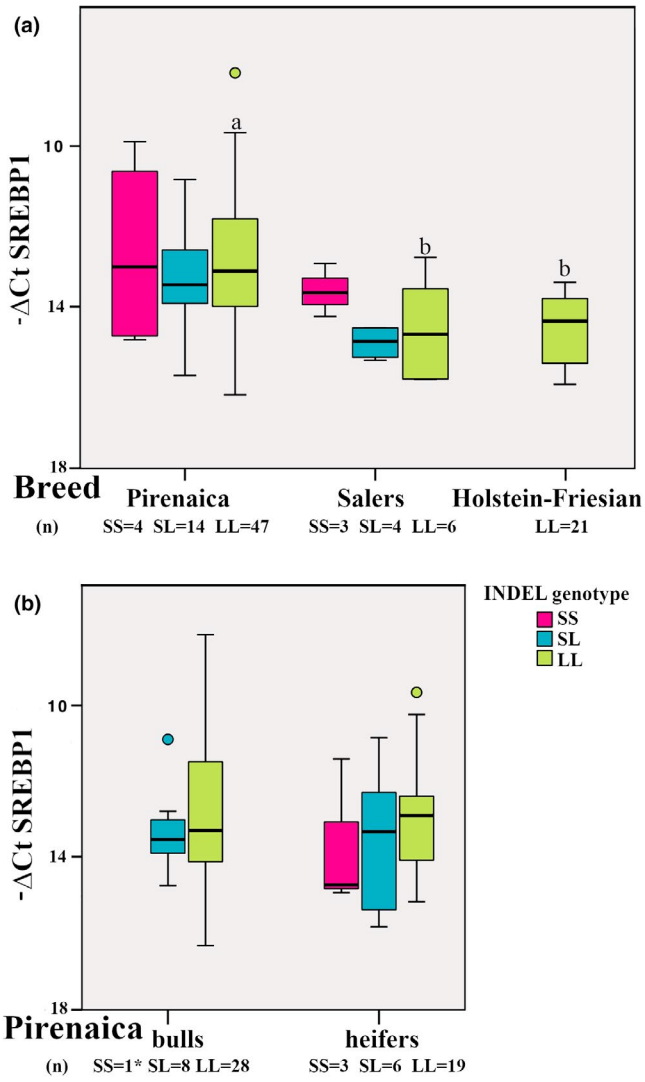

FIGURE 3 Comparison of *SREBP1* gene expression among 84 bp-indel genotypes. (a) Salers, Pirenaica, and Holstein-Friesian breeds and (b) Pirenaica bulls and heifers. *The SS genotype in Pirenaica bulls ($n = 1$) did not permit box plot explanatory analysis

4 | DISCUSSION

The *SREBP1* 84 bp-indel polymorphism has been already discovered and genotyped by a conventional PCR amplification and gel electrophoresis (Hoashi et al., 2007). In the present study, the HRM technique was applied, which is a fast, simple, and highly reliable method developed from previously established genotyping technologies (Liew et al., 2004). An optimized protocol with a new screening method to genotype the insertion/deletion of the polymorphism of interest (84 bp-indel) by Real-time PCR-HRM analysis has been successfully performed (Figure 1).

SREBP1 and *SREBP2* have preferential roles in FA and cholesterol metabolism, respectively (Horton et al., 2002), while gene duplication occurred when independent regulation of FA and cholesterol was required (Osborne & Espenshade, 2009). However, little is known about this issue in bovine and other mammals. The maximum-likelihood mRNA tree of *SREBP1* showed a phylogenetic organization (Figure 2) similar to previous studies performed with mitochondrial DNA of ruminants and mammals (Hiendleder et al., 2008). This study showed that the similarity of *SREBP1* mRNA coding sequence, (Figure 2a), was lower in comparison with the similarity of intron 5 (Figure 2b) in ruminants. This result was unexpected since mRNA is normally translated to protein and usually shows high homology compared to non-coding introns. Although, similarity between *Bos taurus* and *Bos indicus* was the highest (99%), S allele (deletion) was absent in *Bos indicus* sequences (GenBank), which is in agreement with LL genotype observed in Asian zebu breeds (Kaneda et al., 2011). In general, the 84 bp-indel region showed higher similarity than mRNA or intron 5 regions suggesting that this could be a well conserved region throughout evolution with an undetermined functions in *SREBP1* or that even could act regulating transcription levels of downstream genes. Similarly, intron 16 of *SREBP1* has been described to be highly conserved among mammals (Taniguchi et al., 2014), which encodes a non-coding RNA ssc-miR-33 in pigs and bta-miR-33b in cattle (Strozzi et al., 2009). Accordingly, alignment analyses concluded that 84 bp-indel region seems to be highly conserved in mammals and could have some function. However, the absence of S allele in mammals other than *Bos taurus* suggests that the 84 bp-indel polymorphism could have appeared after *Bos indicus* and *Bos taurus* species differentiation, reported to occur 1.7–2 million years ago (Hiendleder et al., 2008).

In terms of bovine commercial types studied, Pirenaica is the most important beef cattle breed in northern Spain, highly appreciated for

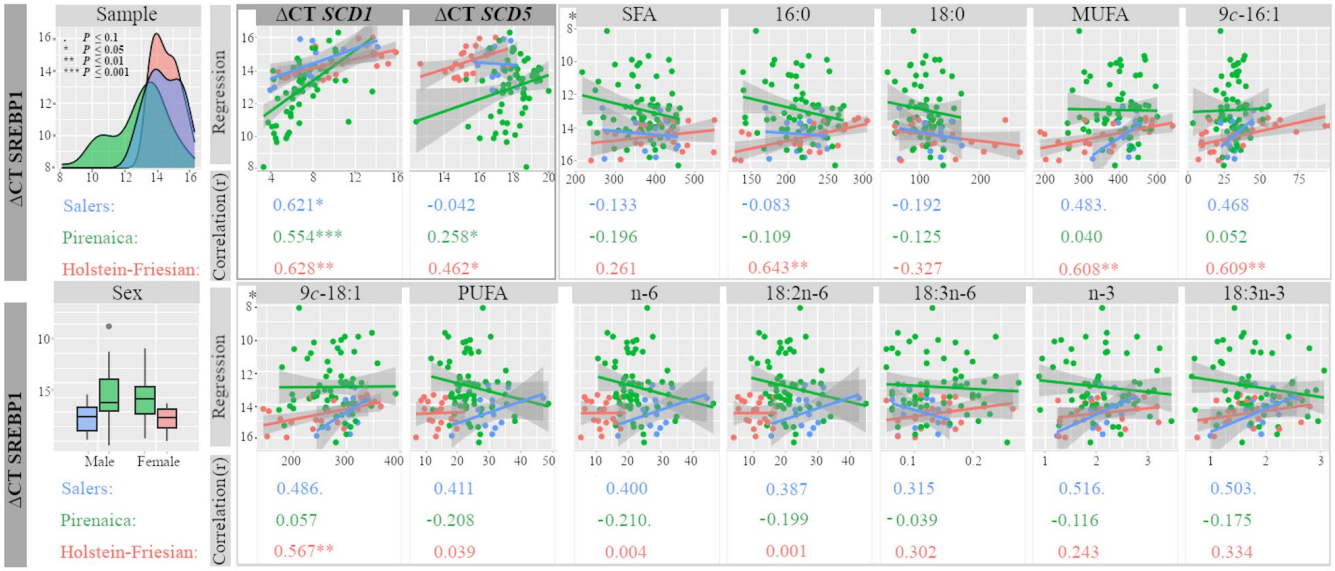

FIGURE 4 Regression and correlations between lipogenic gene expression and FA content of bovine commercial types. Gene expression level and FA contents are represented with ΔCT and mg/g of fat tissue, respectively. *The scale of ΔCT *SREBP1* is inverted to show direct correlation between *SREBP1* gene expression and FA content

its value as a genetic resource as well as for its production traits. But French Salers cattle and Holstein-Friesian cull cows are also an integral part of the regional beef supply. This study characterized the 84 bp-indel polymorphism of the *SREBP1* gene and showed, for the first time, the presence of S allele and SS genotype in Salers and Pirenaica cattle breeds. In previous studies with bigger sample size, only SL and LL genotypes were reported in Simmental and crossbred beef breeds (Barton et al., 2010; Han et al., 2013). The frequency of S allele in Salers bulls was 0.385, higher than Pirenaica heifers (0.214) and bulls (0.135). In contrast, all Holstein-Friesian individuals showed LL genotype as already reported (Huang et al., 2011; Kaneda et al., 2011; Proskura, 2013) which could have been the consequence of a high selection pressure toward milk production. The scientific literature shows that beef breeds seem to have higher S allele frequency (0.450; Kaneda et al., 2011) compared to dairy breeds (0.160; Conte et al., 2010). Interestingly, S allele frequency of Salers was similar to Limousin (0.335; Proskura, 2013) breed, which might be an effect of the phylogenetically and geographically close relationship between the two breeds (Gamarra et al., 2016). In general, these results indicate higher S allele frequencies in European beef cattle breeds, although the highest frequency values were reported in Japanese Black (0.450; Kaneda et al., 2011).

This polymorphism has been associated to FA composition. First, Hoashi et al. (2007) indicated that S allele of the 84 bp-indel contributed to a higher (1.3%) intramuscular MUFA content in Japanese Black. Bhuiyan et al. (2009) reported higher stearic acid (18:0) content in muscle fat of Korean Hanwoo bulls with LL genotype compared to LS and SS genotype. However, in the present study, higher 18:0 content was observed in SS/SL compared to LL genotype in Pirenaica bulls (Table 1). In Pirenaica breed and in terms of PUFA content, the higher content in 18:3n-3 and total n-3 in SL compared to LL genotype may suggest an effect of this polymorphism on PUFA ($p < .05$; Table 2), also corroborated with linear regressions (Table 3). Interestingly, Bhuiyan et al. (2009) also reported higher muscle 18:2n-6 and total PUFA contents in SS compared to LL genotype in Hanwoo bulls. In Pirenaica breed, significant regressions observed may imply a sex-dependent effect of 84 bp-indel. Differences between bulls and heifers should also be considered as a result of a lipogenic gene regulation in a sex-dependent manner (Gamarra et al., 2018). However, *SREBP1* gene expression did not differ among 84 bp-indel genotypes in Pirenaica (Figure 3). Therefore, even 84 bp-indel was associated with several FA contents (Table 2), the mechanism by which it could be related and its relationship with gene expression is not completely clarified.

Holstein-Friesian cows, with only LL genotype, showed different correlation between *SREBP1* expression and FA content compared to the other commercial types studied (Figure 4), that could be related to the older age of these animals, but also their selection towards milk production compared to Pirenaica and Salers, which are specifically raised for meat production.

In previous publications, *SCDs* have been associated with FA composition of adipose tissues (Barton et al., 2010; Gamarra et al., 2018; Kgwatalala et al., 2007; Li et al., 2012; Rincon et al., 2012; Taniguchi et al., 2004). Additionally, SREBP1 can directly activate the expression of over 30 genes, including *SCD* (Horton et al., 2002). As such, significant correlations between *SCD1* and SFAs (including 16:0) were observed in SL genotype of Pirenaica bulls, but also 9c-16:1 in Pirenaica breed (Figure S1a), whereas *SCD5* was negatively correlated with 16:0 ($p < .05$; Figure S1b). However, when the bovine commercial types are not classified by the 84 bp-indel genotype, correlations between *SCD1* or *SCD5* and 16:0 are not significant, but are correlated with other FAs (Figure S3). In a previous study, we reported a novel genetic compensation mechanism between *SCD1* and

SCD5 that showed that one *SCD* isoform could well be compensated by upregulation the other isoform (Gamarra et al., 2018). This opposite pattern has been consistently detected regardless of the genotype of the 84 bp-indel (Figures S1 and S3). Several transcription factors apart from *SREBP1* such as *liver X receptor* (*LXR*), *peroxisome proliferator-activated receptor alpha* (*PPARα*), *CCAAT/enhancer binding protein alpha* (*C/EBPα*), *nuclear factor-1* (*NF-1*), *nuclear factor-Y* (*NF-Y*), and *Sp1 transcriptional factor* (*Sp1*), have also been revealed to bind the *SCD* promoter region (Mauvoisin & Mounier, 2011), therefore they could well be involved in the regulation of *SCD1/SCD5* compensation pattern.

These results suggest that the 84 bp-indel polymorphism is associated with FA composition among studied bovine commercial types, especially in Pirenaica which SL genotype was associated with higher SFA (18:0) and PUFA (18:3n-3) contents. Moreover several differences were observed in correlations between *SCDs* and FAs depending on 84 bp-indel, while the compensation pattern between *SCD1* and *SCD5* was maintained regardless of the genotype of 84 bp-indel. The phylogenetic analysis has demonstrated the high similarity in the 84 bp-indel and flanking regions suggesting the need for its conservation throughout evolution in ruminants. Therefore, the 84 bp-indel region may have an undetermined function in *SREBP1* or may even act regulating transcription levels of downstream genes. Controlled studies will help to elucidate the effect of 84 bp-indel along with lipogenic gene regulation and FA composition.

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CONFLICT OF INTEREST

The authors declare that there is not conflict of interest.

AUTHORS' CONTRIBUTIONS

DG analyzed all experimental data used in this study and drafted the manuscript. NA analyzed and interpreted fatty acid composition data. AA participated in statistical analyses. MT analyzed and interpreted lipogenic gene expression data. MMP participated in the interpretation and discussion of data. All authors contributed to the writing of the manuscript and gave final approval for its publication.

ETHICS APPROVAL

All animals were handled at slaughterhouse following the European Council Regulation (EC) N° 1099/2009. All material was sampled after slaughter and therefore do not require any ethical consideration or approval.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Table S1. GenBank accesion numbers of sequences used for alignment of mRNA and intron sequences.

Species	**mRNA sequence**	**mRNA-bp**	**Genomic sequence**	**Chromosome**	**Intron (bp)**
Bos indicus	XM019980921.1	4152	NC_032668.1	19	35680948-35681569
Bos taurus	NM001113302.1	4443	NC_037346.1	19	34642928-34643549
Canis familiaris	XM022418000.1	4177	NC_006587.3	5	41680271-41680912
Capra hircus	NM001285755.1	3580	NC_030826.1	19	34293114-34293343
Equus caballus	XM027974785.1	4190	NC_009154.3	11	60430347-60431046
Homo sapiens	NM001321096.3	4056	NC_000017.11	17	17818375-17819012
Mus musculus	NM001358314.1	3853	NC_000077.6	11	60205234-60205855
Ovis aries	XM027974781.1	3968	NC_040262.1	11	28668224-28668840
Pan troglodytes	XM016932331.2	4451	NC_036896.1	17	33963057-33963695
Rattus norvegicus	NM001276708.1	3805	NC_005109.4	10	46577429-46578047
Sus scrofa	XM021066226.1	4209	NC_010454.4	12	60744304-60744896

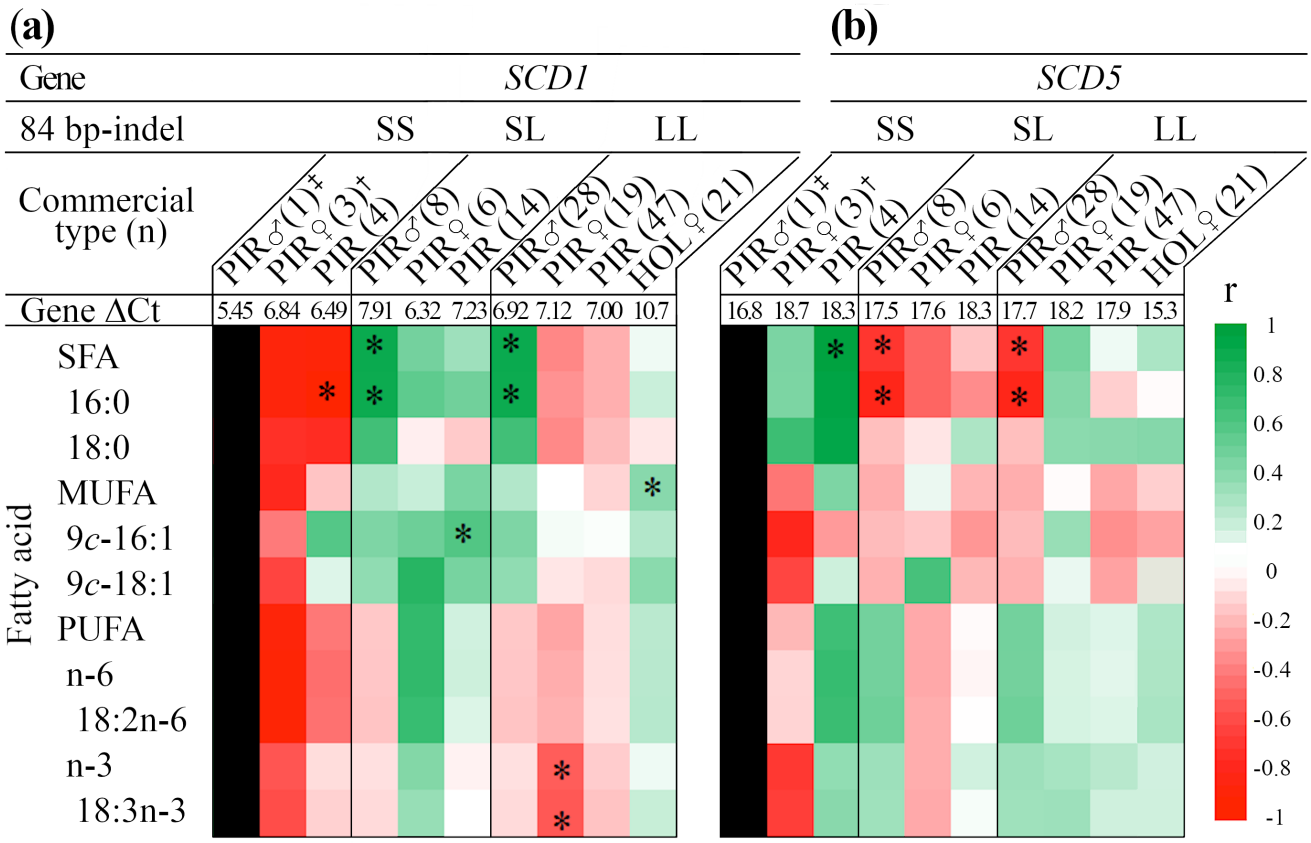

Figure S1. Heatmap matrix of the correlations between the gene expression of (a) *SCD1*, (b) *SCD5* and fatty acid content correlations of subcutaneous adipose tissue of bovine commercial types separated by the 84 bp-indel genotype (SS vs SL vs LL). PIR♂, Pirenaica bulls; PIR♀, Pirenaica heifers; PIR, Pirenaica bulls & heifers; HOL♀, Holstein-Friesian cows. Sample size in brackets. †Correlation analysis performed without GE covariate (*SREBP1* gene expression). ‡No correlation analysis performed.

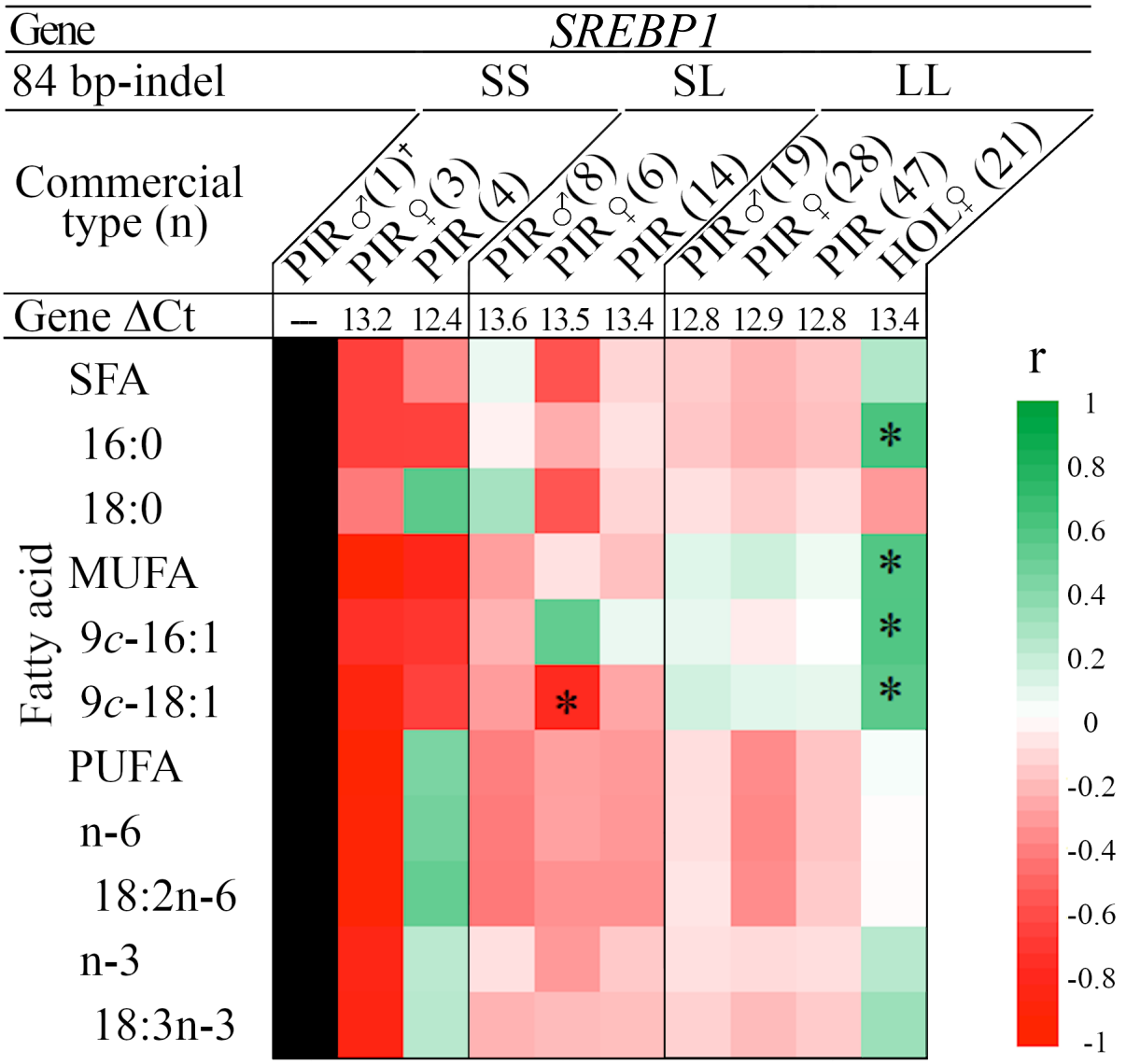

Figure S2. Heatmap matrix of the correlations between the gene expression of *SREBP1* and fatty acid content correlations of subcutaneous adipose tissue of bovine commercial types separated by the 84 bp-indel genotype (SS vs SL vs LL). PIR♂, Pirenaica bulls; PIR♀, Pirenaica heifers; PIR, Pirenaica bulls & heifers; HOL♀, Holstein-Friesian cows. Sample size in brackets. †No correlation analysis performed.

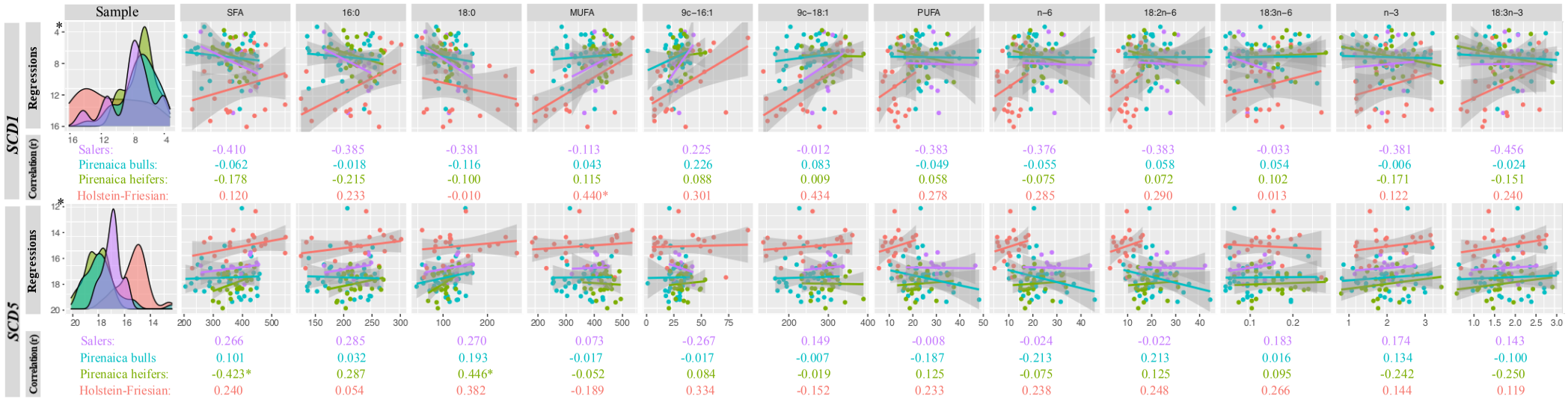

Figure S3. Regressions and partial correlations (Covariate: ΔCT *SREBP1*) between gene expression (ΔCT) of *SCDs* and FA content (mg/g fat) of bovine commercial types. * Scale of ΔCT *SCDs* is inverted to show direct correlation between gene expression and FA content.


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