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ESTUDIO DE MARCADORES SNP ASOCIADOS A CARACTERES DE CALIDAD DE CARNE EN DOS MÚSCULOS, *Psoas major y Flexor digitorum*, EN LA RAZA AVILEÑA NEGRA IBÉRICA

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Memoria presentada por Ximena Quintero Arboleda para optar al grado de máster por la Universidad Politécnica de Valencia

Las Directoras,

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RESUMEN

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Este estudio tuvo como objetivo identificar marcadores genéticos asociados a la grasa intramuscular (IMF), el flavor (FLA), fuerza de corte (SF) y la terneza de la carne (TER) en dos músculos diferentes, el *M. psoas mayor* (PM) y el *M. flexor digitorum* (FD) . Un total de 397 terneros de la raza Avileña - Negra Ibérica fueron genotipados con la plataforma Illumina Bovine SNP50, para este estudio se tomaron medidas de grasa intramuscular (%), la fuerza de corte con la medida de compresión Warner- Blazter (CMB) y dos caracteres evaluados por paneles de degustación que incluían puntuación para terneza y para el flavor. Se llevó a cabo una pre-selección de los SNP contenidos en 42 regiones QTL identificados previamente como asociado con el IMF, 19 en regiones QTL para la FLA y 140 en regiones QTL para SF-TER descritas en la base de datos del Genoma Animal UMD_3.1. Un estudio de asociación de genoma completo (GWAS) se realizó mediante QXPak 5.2. Se utilizó un modelo que incluye el lugar-año y la duración del engorde, la edad, el lugar y la temporada del sacrificio, sesión de cata y catador (sólo para FLA y TER), evaluando el SNP en el momento y el efecto aditivo poligénico. Un total de 1.138, 2.265 y 3.468 SNPs fueron analizados para IMF, FLA y SF- TER respectivamente. El GWAS reveló que 2/3, 2/0, 5/0 y 3/0 SNP tenían un efecto significativo ($P < 0,0001$) en al IMF, FLA, SF y TER respectivamente, en FD/PM. Los SNP identificados han permitido la validación de doce QTL que se han descrito anteriormente en otras poblaciones. Se definieron cinco regiones de desequilibrio de ligamineto (LD) para IMF, dos para FLA, cinco para SF y tres para TER utilizando un umbral $LD = 0.2$, a partir de las cuales se identificaron una serie de genes relacionados con el FMI, FLA, SF y RTE, así como también se han propuesto una serie de ellos.

SUMMARY

This study aimed at identifying genetic markers associated with intramuscular fat (IMF), flavor (FLA), shear force (SF) and beef tenderness (TER) in two largely different muscles, Psoas major (PM) en Flexor digitorum (FD). A total of 397 Avileña-Negra Ibérica calves with genotypes for the Illumina Bovine SNP50 platform and measures of intramuscular fat (%), shear force with Warner-Blazter compression measure (WBC) and traits evaluated by trained sensory panels that included tenderness score and flavor participated in the study. A pre-selection of SNPs was carried out by determining the SNPs contained in 42 QTL regions previously identified as associated with IMF, 19 QTL regions for FLA and 140 QTL regions for SF-TER in the Animal Genome UMD_3.1 database. A genome wide association study (GWAS) was then performed using QXPak 5.2. A model including place-year and length of fattening, age, place and season of slaughter, panel session and panelist (only for FLA and TER), one SNP at a time and the polygenic additive effect was used. A total of 1138, 2265 and 3468 SNPs were including in analysis for IMF, FLA and SF-TER respectively. The GWAS revealed that 2/3, 2/0, 5/0 and 3/0 SNPs were a significant ($P < 0.0001$) effect on to IMF, FLA, SF and TER respectively in FD/PM. The SNPs have allowed validation of twelve QTLs that have been previously described in another type of populations. Define five desequilibrum of linkage (LD) regions for IMF, two for FLA, five for SF and three for TER using a DL=0.2 threshold, were are identified a number of candidate genes related to IMF, FLA, SF and TEN as well as also have proposed a number of them.

INTRODUCIÖN

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Tanto en España como en los países de nuestro entorno, se ha observado en los últimos años una reducción en el consumo per cápita de la carne de vacuno a menos de 15 Kg por habitante por año. Esta tendencia ha sido atribuida en gran medida a la crisis económica actual pero por otro lado también se atribuye a la percepción que el consumidor tiene de este tipo de carne (Troy y Kerry, 2010) y que además es más cara que otras fuentes de proteína que resultan más asequibles (MAGRAMA, 2013). Dicha percepción por parte del consumidor se ha producido, según Eggen and Hocquette (2004) por múltiples factores, tales como la inconsistencia en las características sensoriales, el alto contenido en grasas saturadas y la preocupación creciente del consumidor por el bienestar animal.

Por otro lado, el sector de vacuno de carne ha tenido que soportar algunas crisis sanitarias (AgraEurope, 2001), lo cual ha generado que un porcentaje apreciable de consumidores estén dispuestos a adquirir carne de calidad certificada, de esta manera se abre un nicho de mercado que justifica la búsqueda vía selección de la mejora de la calidad de carne (Diaz *et al.* 2006; Martín-Collado *et al*. 2012). Este interés es mayor para los productores de aquellas razas que requieren del valor añadido que la calidad puede dar al producto final, puesto que de ésta forma es posible compensar el menor valor de la canal castigada por tener peor conformación cuando se compara con razas mas seleccionadas (Martín-Collado *et al*. 2012). Este es el caso en particular de la raza Avileña Negra-Ibérica, la cual se encuentra posicionada como la tercera mayor productora de carne en cuanto a razas autóctonas de España. Para el Consejo Regulador de la IGP (Consejo Regulador I.G.P. Carne de Avila, 2012) la raza Avileña-Negra Ibérica con sello de Identificación Geográfica Protegida, se encuentra presente en más de 700 explotaciones situadas en 19 provincias y 7 Comunidades Autónomas y dispone de un censo de 21.100 reproductoras en activo y bajo control.

Existen diversos parámetros que indican la calidad de la cerne tales como el valor nutricional, las características sanitarias, las características intrumentales, entre otros, pero en relación a la calidad de las carnes bovinas, es muy posible que el aspecto más importante sea el conocimiento de aquellos factores que determinan la preferencia del consumidor ya que este es el actor principal dentro del sistema global de producción y comercialización de la carne, por lo tanto resulta imprescindible tener caracterizados los parámetros o atributos que inciden sobre la elección del producto al momento de su adquisición y consumo (Teira *et al*, 2006).

A pesar de que las necesidades, preferencias e incluso la información de la cual disponen los consumidores son sumamente variables, de acuerdo con Miller (2003) (citado por Teira *et al*. 2006), tales características se pueden agrupar de la siguiente manera: apariencia visual (color de la carne y de la grasa subcutánea, firmeza o consistencia, textura, cantidad de grasa extra-muscular, marmorización y exudado), calidad comestible (jugosidad, terneza, aroma, sabor) y otros factores que podrían incluir el precio, tamaño de la porción, facilidad y forma de preparación, envasado e información sobre el valor nutritivo, de salud y seguridad. Es importante también tener en cuenta que la calidad de la carne es un carácter complejo, influido por un gran número de factores como la bioquímica del músculo (Moreno-Sanchez *et al.* 2008), la composición fibrilar, la raza (Laborde *et al.* 2001; Moreno-Sanchez *et al*. 2008), el sexo o las condiciones de sacrificio (Insausti *et al*. 2008). Un ejemplo sobre la influencia de múltiples factores en la calidad de la carne, se puede encontrar en estudio realizado a carcazas de añojos de raza Avileña Negra Ibérica y de cruces (Panea *et al.* 2012), donde se determinó que tanto el tipo genético como el sexo de los animales tuvieron influencia sobre la mayoría de las variables estudiadas, pero las diferencias entre los individuos puros y cruzados (tipo genético) dentro de sexo (machos) fueron menores que entre sexos (machos y hembras) dentro de tipo genético (animales cruzados). En general, la carne de las hembras fue mejor valorada que la de los machos. La maduración incrementó la jugosidad, la terneza de la carne y se intensificó el olor y el flavor.

Desde el punto de vista del consumidor, la percepción sensorial de la carne es uno de los aspectos más relevantes a tener en cuenta, como lo describe Martín-Collado y

Díaz (2012) en el estudio que realizaron encuestando a 384 personas pudiendo determinar la importancia relativa de los componentes sensoriales de la calidad de la carne desde el punto de vista de los consumidores los cuales categorizaron el sabor como el componente sensorial más importante seguido de la terneza de la carne.

Es importante tener en cuenta que estos caracteres se comportan de forma dependiente entre ellos lo que hace complicado tener una valoración objetiva e independiente de cada uno, por ejemplo, el conjunto de percepciones gustativas y olfatorias se denomina flavor (FLA), siendo un carácter que solo se puede apreciar si hay una consistencia entre la terneza y la jugosidad (Renand *et al.* 2006), por otro lado caracteres cuantitativos como el engrasamiento de la carne afectan significativamente la valoracion sensorial ya que repercute directamente en la jugosidad y el flavor (Osoro *et al.* 2006; Dubost *et al.* 2013), en cuanto a la terneza (TER) de la carne hay estudios que confirman que esta estrechamente relacionada con la la grasa intramuscular (Barendse *et al.* 2011; de Jager *et al.* 2013), sin embargo la relación que existe entre fuerza de corte (SF), la grasa intramuscular (IMF) y la terneza (TER) pueden varíar en función de la raza y la preparación de la muestra (Christensen *et al.* 2011). La relación que guardan estos caracteres también se pudo reflejar en un estudio que realizaron Díaz *et al.* (2006), donde se observaron las diferencias de las medias de la distribución marginal posterior entre el músculo *Flexor digitorum* (FD) y *Psoas major* (PM) en un modelo animal de repetibilidad dando como resultado un aumento en la diferencia de la terneza cuando se ajustaba por grasa intramuscular (IMF), mientras que con este mismo ajuste, las diferencias de FLA y resistencia al corte (SF, medida como warner bratzer) disminuían, siendo mas relevante la disminución de la diferencia en cuanto a resistencia al corte (SF) que pasó de 1.40 a 0.78, cerca del 44% menos. Sin emabargo, dicha relación cuando se refiere a la resistencia al corte en carne cruda, es dependiente del músculo analizado (López de Matura *et al.* 2010), estos autores encontraron que la terneza en el músculo PM estaba asociada al contenido de IMF, mientras que en el músculo FD parecía estar asociado al contenido en colágeno. Sin embargo, esta última relación parece depender de la forma en que se determina SF ya sea en carne cruda y/o en carne cocinada (Torrescano *et al.* 2003; Christinesen *et al.* 2013). En lo que se refiere al componente genético, la genética cuantitativa clásica ha demostrado la existencia de

diferencias en la calidad de la carne entre y dentro de las razas bovinas, al margen de estas también se conocen claras diferencias de calidad entre piezas de una misma canal resultado de una variabilidad propia del animal explicada por una base genética que actúa en función de las necesidades físicas y metabólicas de los músculos como lo describen Moreno-Sánchez *et al.* (2011), en un estudio de expresión diferencial de genes que realizaron en AVI, donde detectaron 204 genes que se expresaban de forma diferencial en cada músculo (*Flexor digitorum* y *Psoas major*), información de gran importancia dado que la mayoría de los estudios de calidad de carne con marcadores han sido realizados en el musculo Longissimus dorsi y poco se ha estudiado en otros músculos.

Por otro lado la evolución de los métodos y herramientas usadas para la evaluación y selección de animales de producción ha sido muy rápida en las últimos años, creando una nueva tendencia en el manejo de los programas de selección que no descartan el uso de métodos clásicos como las pruebas de progenie (MacNeil *et al.* 2010) combinados con el uso de la información genómica más moderna, siendo esta combinación una herramienta prometedora para aumentar la respuesta a la selección que ayuda en la predicción de los valores genómicos directos y que aumenta la precisión de la predicción del valor genético de los animales a una edad temprana (Saatchi *et al.* 2012).

Hay que recordar que en la mejora genética clásica los datos usados son básicamente el parentesco y el fenotipo analizado con el modelo más usado que es el modelo infinitesimal, este modelo asume que la varianza de un carácter esta dada por el efecto aditivo de infinitos loci no ligados y con efecto infinitesimal de cada uno (Fisher, 1919), sin embargo existe el modelo de loci finitos que asume un numero finito de loci interviniendo en la varianza de un carácter, incluso existen trabajos que demuestran que en la distribución de los loci hay unos pocos con un efecto grande y muchos con un efecto muy pequeño (Shrimpton y Robertson, 1988; Hayes *et al.* 2001), y cabe mencionar que este último es la base de los modelos de búsqueda que pretenden incrementar la precisión de los valores de mejora usando la información molecular de las diferencias en las secuencias del ADN. El uso de la información molecular para la realización de mejora genética se basa en dos estrategias, la primera es la de el “Gen

candidato", que intenta explicar la varianza de un carácter por las mutaciones que tenga un gen el cual debe estár fisiológicamente asociado al carácter o "genes candidatos posicionales" los cuales están en regiones del genoma identificadas que influyen en un fenotipo, con esta estrategia se han propuesto genes candidatos como el gen DGAT1 estudiado en el músculo Semitendinosus de las razas holstein, charoláis y razas chinas comerciales y el gen TG estudiado en el músculo Longissimus dorsi de las razas holstein y charoláis, donde se encontró que estos dos genes están asociados a IMF (Thaller *et al.* 2003; Yuan *et al.* 2012), por otro lado el gen de la Myostatina también ha sido asociado a IMF y TEN, este gen se ha estudiado en individuos cruzados de Belgian Blue × MARC III ($^1/_4$ Angus, $^1/_4$ Hereford, $^1/_4$ Red Poll, $^1/_4$ Pinzgauer) y de Piedmontese × Angus (Casas *et al.* 1998, 2000) con el fin de conocer el efecto de las variantes alélicas de este gen sobre el fenotipo, otro ejemplo de son los genes CRYAB y PDLIM3 identificados como significativamente asociados a la grasa intramuscular e incluso expresados diferencialmente en el músculo Longissimus dorsi en un estudio realizado en siete razas bovinas (Reverter *et al.* 2008), un ejemplo más de genes identificados asociados a caracteres de calidad de carne son el CAPN1 y CAST genes de gran importancia que forman parte del sistema calpaina-calpastatina determinantes de características durante la maduración postmortem de la carne (E Huff-Lonergan *et al.* 1996) y de los cuales se conoce su actividad en la tiernización de la carne por lo que se han realizado numerosos estudios con el fin de detectar y validar marcadores sensibles de las variaciones de CAPN1 (Page *et al.* 2002; S. N. White *et al*. 2005) y CAST (Casas *et al.* 2006; Barendse *et al.* 2007; Motter *et al.* 2009; Allais *et al.* 2011; Iguácel *et al.* 2013).

La segunda estrategia para el uso de la información molecular, es la del mapeo de QTL (Quantitative Trait Loci), en español "Locus para una característica o rasgo cuantificable", de esta forma se identifican regiones asociadas a las variaciones de los fenotipos, esta técnica, asume que no se conocen los genes que afectan el carácter, y se realiza analizando la asociación entre la variación alélica de un marcador y la variación del fenotipo, al detectar asociación significa que el marcador esta asociado al fenotipo.

Desde principios de la década de los 90´s se ha realizado una búsqueda intensiva de QTLs, en distintas razas de vacuno de carne y sus cruces (www.animalgenome.com), en la base de datos de The National Animal Genome Research Program se enumeran alrededor de ocho mil QTLs en ganado vacuno que representan cerca de cuatrocientos setenta caracteres descritos en cuatrocientos cincuenta publicaciones aproximadamente y de los cuales el 65% de los QTLs descritos para carne se refieren a la calidad. En concreto para el carácter grasa intramuscular (IMF) se encuentran cuarenta y dos QTLs reportados en trece publicaciones, para flavor (FLA) se cuenta con diecinueve QTLs agrupando los QTLs de los caracteres sabor, intensidad de olor, intensidad de sabor, sabor anormal y olor anormal, reportados en cuatro publicaciones de estudios realizados con marcadores microarrays (Alexander *et al.* 2007; Gutiérrez *et al.* 2008) y otros realizados con marcadores de un solo nucleótido polimorfico (SNP) (Gil *et al.* 2009; Reardon *et al.* 2010). En lo que se refiere a TEN y SF en conjunto, existen alrededor de 140 QTLs que agurupan cerca de dieciséis caracteres descritos.

Los QTLs se pueden describir entonces como regiones del genoma responsables de la expresión fenotípica de los caracteres quantitativos. Inicialmente, dicha búsqueda de QTLs se realizó utilizando marcadores microsatélites con diferentes densidades permitiendo identificar regiones de interés potencial a lo largo del genoma, cabe mencionar que actualmente la tendencia es el uso de marcadores SNPs. La detección de QTLs se basa en el supuesto de que dichos QTLs están en desequilibrio de ligamiento (DL) con una serie de marcadores flanqueantes disribuidos a lo largo del genoma. La determinación del ligamiento entre QTL y marcadores se realiza mediante un análisis de asociación y de ligamiento basado en la posición de los marcadores con el fin de capturar la señales generadas por regiones del genoma para la posterior búsqueda de genes candidatos por su funcionalidad y rutas metabólicas en las que intervienen. La información obtenida de los QTLs es una herramienta de gran ayuda a la hora de hacer selección ya que se puede llevar a cabo la genotipificación de individuos en las poblaciones de especies animales, entre los que se puede detectar a aquellos que son portadores de estos marcadores para ser seleccionados y de esta manera desarrollar programas de mejora basados en las regiones QTLs para los caracteres de interés (Dekkers, 2004; FAO, 2003).

Para los caracteres grasa intramuscular, flavor, resistencia al corte (también llamada Shear force (SF) y medida como Warner-Bratzer) y terneza se los QTLs descritos están distribuidos en diferentes cromosomas, en algunos casos estas regiones definidas por los QTLs incluyen genes que podrían ser responsables en gran medida, del carácter al que hacen referencia, sin embargo una de las dificultades que existe actualmente en el uso de los QTLs para la búsqueda de genes candidatos es que algunos QTLs llegan a comprender regiones muy extensas y densas de genes que dificultan la búsqueda o que los marcadores asociados a estos no sean consistentes en todas las poblaciones, por lo que es recomendable profundizar en el uso de herramientas para disminuir e identificar regiones de tamaño reducido que puedan reflejar el mismo efecto y se ajusten a la estructura de la población con el fin de facilitar la búsqueda de genes o grupo de genes que determinan parte de la variación del un carácter.

A día de hoy ya se usan herramientas de selección que combinan los métodos tradicionales con información molecular entre los cuales se puede mencionar MAS (Marker Assisted Selection), con estrategias que se basan en el desequilibrio de ligamiento (DL-MAS y LE-MAS), sin embargo la selección genómica se propuso como una alternativa a estos métodos (Meuwissen *et al.* 2001), siendo la selección genómica un método más completo al incluir todos los QTLs y no un numero limitado, ya que dada la cantidad de marcadores disponibles, se puede cubrir todo el genoma garantizando que todos los QTLs están en desequilibrio de ligamiento con uno o varios marcadores. actualmente, gracias al desarrollo y avance tecnológico se cuenta con chips de genotipado que contienen más de cincuenta-mil marcadores SNP (Single Nucleotide Polymorphism) caracterizados en diferentes poblaciones y razas de ganado vacuno (Van *et al.* 2008), lo cual equivale a una densidad de quince a veinte marcadores por cada millón de pares de bases (Mb) aproximadamente.

Son entonces los SNP los actuales marcadores de selección genómica y se refieren al cambio de una base nucleotídica en la secuencia del ADN, esta identificación de polimorfismos en nucleótidos simples (SNP) permite que los genetistas puedan estudiar la posible asociación de los mismos con los caracteres de importancia económica

(Moskvina & Schmidt, 2008). Para los caracteres de calidad de la carne, existen varios marcadores tipo SNP que han sido identificados (Page *et al.* 2004, Bolormaa *et al*, 2011), esta información molecular permite realizar selección directamente por el genotipo, lo que es muy valioso en rasgos cuantitativos de difícil medición como es el caso de la terneza.

Se ha estimado que el genoma bovino consta de 2.87 Gb osea, casi tres millones de pares de bases (Elsik *et al.* 2009), esto demuestra la gran problemática que puede existir con la identificación de la asociación entre los marcadores de tipo SNP y los caracteres cuantitativos ya que en total se cree que hay aproximadamente un SNP cada 300 – 500 bases en el genoma, y teniendo en cuenta que la proporción de la varianza genética explicada por un SNP asociado es baja, se reduce la probabilidad de identificar SNP que aporten un efecto significativo al fenotipo lo cual se compensaría solo si el tamaño de la muestra fuera lo suficientemente grande para poder generar un mayor poder de detección (Peter M. *et al.* 2012). La complejidad de los análisis en este tipo de estudios reside en que al ser un estudio de múltiples pruebas, las asociaciones deben repetirse para miles de SNPs probados. La p convencional, es decir, menor de 0.05, en estos estudios mostrarán cientos o miles SNPs asociados al carácter, de los cuales casi todos son falsos positivos. Por esto puede ser recomendable aplicar correcciones como la de Bonferroni, en la cual los valores de p convencional se dividen por el número de SNPs analizados es decir, en un análisis de 50 mil SNPs se debe usar un umbral de $p<0.05/5x10^4$, también se han propuesto otros tipos de análisis que incluyen el estimado de la tasa de falsos positivos el cual depende del tamaño muestral de tal manera que a medida que la muestra es mas grande la tasa de falsos positivos disminuye (Bolormaa *et al.* 2011), la probabilidad del reporte de falsos positivos, la probabilidad de que la hipótesis nula sea verdadera dando una significancia estadísticamente significativa y el estimado de los factores del teorema de Bayes (Zhang Y, Liu JS 2007), es decir, incorporar la probabilidad a priori de la asociación basada en las características del carácter o del SNP específico (McCarthy MI *et al.* 2008), cabe mencionar que el ajuste por Bonferroni es la más usada en los GWAS (Pearson *et al.* 2008, Zhang *et al.* 2012).

Otra controversia que se ha generado en el estudio con GWAS es que se asumen asociaciones independientes de cada SNP con los fenotipos, sabiendo que teóricamente dichos SNPs están correlacionados en algún modo debido al desequilibrio de ligamiento, (Yang Q *et al.* 2005; Pearson TA *et al.* 2008). Según un estudio realizado por Daniel Gianola (2013), la proporción de la varianza de un carácter explicada por un loci está plenamente afectada por el desequilibrio de ligamiento y depende en gran medida de la covarianza con otros loci, ósea que la parte de la varianza aportada por loci específicos no es tan sencilla cuando existe desequilibrio de ligamiento, en cambio se requiere el conocimiento de la distribución de los efectos de las frecuencias alélicas además de toda la estructura de desequilibrio de ligamiento para tener una mayor precisión al atribuir dicha varianza. Este tema puede llegar a ser tan delicado e importante a la hora de realizar estudios como el presente, dado que la varianza aportada por un solo QTL o SNP en un análisis GWAS estándar puede ser engañoso tanto conceptual como estadísticamente, cuando el carácter es complejo y depende de muchos genes.

Cuando se detectan marcadores asociados con un carácter que se pretende utilizar para la selección genómica o para el mapeo de QTL en una región cromosómica, es necesario confirmarlos en otras poblaciones, a menudo, estos intentos de confirmación no tienen el éxito esperado (Pryce *et al.* 2010), esto puede suceder porque el descubrimiento original era un falso positivo, porque la asociación es específica y no se refleja ya sea porque el QTL no segrega en otra raza o porque el LD difiere entre razas, o finalmente por falta de potencia estadística, ya sea en el descubrimiento, en la población de validación o de ambas poblaciones (Bolormaa *et al.* 2011), esto significa que aunque un alelo asociado a un carácter pudiese encontrarse asociado igualmente en otra población, es posible que para otra población que difiera en el LD entre marcadores (de Roos *et al.* 2008, 2009), se pudiese observar un LD entre SNPs y QTLs no esperados y por tanto detectar otros SNPs asociados al QTL, esta situación sugiere que para validar la asociación que hay entre los marcadores y los genes o QTLs se debe tener en cuenta la estructura de la población (Gómez, 2012) y mas específicamente las regiones LD del genoma.

Para finalizar, cabe destacar que estudios como este se vienen realizando en los últimos años y cada vez más se acercan a su objetivo principal que es generar herramientas que aumentan la eficiencia de la selección de animales por caracteres tanto productivos como de interés para el consumidor y que puedan ser usadas en diferentes poblaciones. Hemos llevado a cabo un análisis de todo el genoma con marcadores, a partir de regiones QTL en una población con el fin de localizar marcadores SNP que influyen en los dos caracteres sensoriales de calidad de carne mas importantes para el consumidor (flavor y terneza) medidos de forma subjetiva, para tener una visión mas global, nuestro análisis también incluyó dos caracteres muy relacionados con los mencionados anteriormente y que aportan mediciones cuantitativas (grasa intramuscular y resistencia al corte), para maximizar la veracidad de la información obtenida de los resultados se estudió la estructura de la población determinando el perfil de desequilibrio de ligamiento y finalmente teniendo en cuenta las diferencias que existen entre músculos, este análisis se realizo de forma independiente para el músculo *Flexor digitorum* (FD) y el *Psoas major* (PM) de tal manera que se pudiese demostrar la dependencia que existe entre los marcadores asociados a los caracteres de calidad de carne en cada músculo.

OBJETIVE

OBJETIVE

The objective of this work was to deepen into the genetic basis of tenderness, flavor instrumental texture, and intramuscular fat and their differences between *Flexor digitorum* (FD) and *Psoas major* (PM) muscles in Avileña Negra-Ibérica (ANI) calves as taken .

To achieve this general goal we undertook two specific objectives:

1.- To explore in the Avileña Negra-Ibérica population, meat quality QTLs that have been already described in other beef cattle populations. To do so, we have used a set of SNP markers located in those regions.

2.- To use the Linkege Disequilibrium (LD) existing in this population to redefine QTLs associated to the traits of interest.

MATERIALS AND METHODS

MARERIALS AND METHODS

1.- Phenotypic data

1.1 - Animal and meat samples

Meat quality information was available in 397 ANI male calves. Those calves were raised under an extensive production system till weaning. They were weaned between 5 to 8 months of age, and then fattened in eight different feedlots during an average period of 196 ± 48.44 days. After the fattening period, calves were transported to the commercial EU-licensed abattoirs, slaughtered and dressed according to commercial practice. The average slaughter age and slaughter weight were 425.71 ± 54.03 days and 501.54 ± 40.45 Kg, respectively, with an average daily gain of 1.32 kg/d (±0.24).

Samples of FD and PM muscles were taken after carcasses were chilled for 24. Muscles were vacuum-packed and kept 7 days at 4ºC for ageing. Samples were then stored at -20ºC± 2ºC until laboratory analyses and panel tasting were performed.

1.2 - Meat quality phenotypes

To determine these measurements, the samples were first thawed overnight at 4ºC±1º. As it has been mentioned, the available laboratorial measurements for both muscles were intramuscular fat (IMF) and Warner-Blatzer shear force (SF). In addition, two sensorial traits, flavor (FLA) and tenderness (TEN) were also considered.

IMF was estimated with an Infratec 1265 Meat Analyser. Average of thirty transmittance measurements taken from 200 g of homogenized sample placed on a tray, were computed (Josemaría-Bastida, Cruz-Sagredo & García-Cachán, 1999).

SF, measured as the maximum force (kg), was evaluated in at least three manually prepared strips of 1 cm^2 cross section and three to four cm in length, with fibres perpendicular to the direction of the blade attached to an Aname TA-XT2 texturometer (Honikel, 1998).

Samples from FD and PM muscles were prepared differently for the taste panel since regular cooking of these two muscles always differs. After thawing the samples, PM steaks (2 cm thick) were cooked in an electric air-convection oven (preheated at 220ºC for 10 min) until the samples reached 70ºC, measured with a thermocouple in the approximate geometric centre of each steak. FD steaks were cooked in a pressure cooker, and once the boiling temperature was reached, steaks were maintained 7 minutes. Each steak was trimmed of any external connective tissue, cut into 2 cm^2 sections, wrapped in codified aluminum paper and maintained hot until tasted. A ten-member trained panel (UNE87024-1, 1995; UNE87024-2, 1996) assessed the sensory attributes of tenderness, and flavor using a seven-point descriptive scale (1 for the lowest attribute intensity and 7 for the highest one). Table 1 shows the number of animals, mean and other summary statistics for each trait in each muscle.

Table 3. Number of calves samples (N) and summary statistics for the traits analyzed in the two muscles.

Traits[1]	**IMF**		**SF**		**FLA**		**TEN**	
Muscles [2]	**FD**	**PM**	**FD**	**PM**	**FD**	**PM**	**FD**	**PM**
N	390	391	351	357	394	393	394	393
Mean	1.13	2.76	7.86	6.53	3.99	5.28	3.49	5.68
sd	0.81	1.40	4.70	3.44	1.18	1.09	1.40	1.21
Minimum	0.01	0.40	1.73	1.06	1.00	1.00	1.00	1.00
Maximum	6.80	8.80	32.67	32.05	7.00	7.00	7.00	7.00

[1]IMF: Intramuscular fat (%); SF: Warner-Bratzler shear force (kg); FLA: Flavor (score 1 to 7); TEN: Tenderness (score 1 to 7). [2] FD: *Flexor digitorum*; PM: *Psoas major*

2.- Genotypic data

One of the problems we had to face for these analyses was the limited sample size. Thus, we decided to approach the analysis considering different subsets of markers selected attending two biological criteria to be able to accomplish our objectives. Therefore, we first investigated the existing QTLs found in other beef cattle populations and later on we focused on genomic regions where DE genes between the two muscles had been previously detected in this breed (Moreno-Sánchez, 2011). To extract the information we needed for the analysis a suit of programs written in AWK and R (R Development Core Team, 2011) were prepared.

2.1 - SNPs markers

Three hundred ninety seven ANI calves were genotyped with BeadChip BovineSNP50. This platform has 54,609 markers. In total, a file containing 21,679,773 records was generated. Call rate per individual was above 99.9 %, therefore, none of the individuals were removed from the genotype file. Call rates by marker were examined and then those with a call rate below 98% were discarded from the file. Furthermore, markers with a minimum allele frequency equal to 0 were also removed from the file. Thus, 4,14% of markers, specifically, 2263 makers were eliminated because more than 2% of animals were not able to get a genotype for those specific markers; in addition, 11.8% of markers were fixed and therefore removed from the genotypes file. In total, 280 were markers that were fixed with a call rate below 98%. Finally, 46,221 markers were kept for subsequent analyses.

2.2. - Known QTLs regions associated to meat quality traits

In order to pursue the first objective of the thesis, the whole QTLs database from the Animal Genome Project was downloaded in gff format in the bovine UMD3.1 ensemble (www.animalgenome.org). This database currently contains for meat quality, around of 120 entries for 1250 QTLs approximately for 103 traits. This QTLs related to the traits of interest were extracted with a procedure in AWK. Instrumental Texture measurements and sensorial tenderness were grouped under the same group of traits. Thus, the chromosome, the starting and finishing position of 201 QTLs out of the 1257

meat quality QTLs available in the database at the moment of extraction, were obtained. Table 2 shows the number of QTLs for each type of trait as well as the average size of the published QTLs.

Table 4. Number of published QTLs, average size, standard deviation (sd), minimum and maximum size in 1 Mb (1 million base pair).

Traits[2]	**Number of QTLs**	**Average size**[1]	**sd**	**Min. size**[1]	**Max. size**[1]
IMF	42	1.500	4.733	0.001	26.372
FLA	19	6.668	7.911	0.127	23.093
SF and TEN	140	2.199	6.485	0.055	49.770
Total	201	4.806	10.109	0.001	78.988

[1] (1 million base pair = 1 Mb). [2]IMF: Intramuscular fat; FLA: Flavor; SF: Warner-Bratzler shear force; TEN: Tenderness.

Tabla 3. Number of QTLs regions, raw number of SNPs, filtered SNPs located within the chosen regions and minor allele frequency.

Traits[1]	**Number of SNPs**	**Filtered**[2] **Number SNPs**	**MAF**
IMF	1359	1138	0.001259
FLA	2670	2265	0.001259
SF and TEN	4101	3468	0.001259
Total	8130	6871	

[1] IMF: Intramuscular fat; SF: Warner-Bratzler shear force; FLA: Flavor; TEN: Tenderness.[2] SNPs with MAF > 0 and call rate >98%.

A program in R (R Development Core Team, 2011) was written to obtain all SNPs located within each QTL region depending upon the trait. Thus, we generated three genotype files, containing SNPs found within Tenderness (including SF and TEN QTLs), IMF and FLA QTLs, respectively. Table 3 describes the number of QTLs and the number of SNPs as well as the number of SNPs entering the analysis for this first set of makers.

3.- Statistical Anlysis:

To perform the association analysis, each set of markers in QTL regions (in Table 3), were used independently for each corresponding trait in each muscle. Then, markers located in the DE genes regions were analyzed separately to detect associations with each trait.

The model of analysis was as described in the following model equation:

$$y_{ijkl(mn)op} = FedY_i + LFc_j + SAg_k + SSe_l + (SPa_m + Pan_n) + gSNP_o + a_p + e_{ijkl(mn)op}$$

, where $FedY_i$ represents the combination between year and feedlot (19 levels), $+ LFc_j$ is the length of fattening period grouped into three classes (≤162 d; 162-225 d; and >225 d), SAg_k or slaughter age, grouped into three classes (≤390 d; 390-454 d; and >454 d), SSe_l, is slaughter season (3 levels), and *M* which corresponds to slaughterhouse (3 levels). In addition to these effects, the model for sensory traits also included session (SPa_m, 23 levels) and panelist (Pan_n, 10 levels). Furthermore, all model contained, the effect of marker $gSNP_o$, a polygenic effect a_p, and $e_{ijkl(mn)op.}$ the corresponding residual term.

Models were run for each trait in each muscle and each corresponding marker set. to estimate the polygenic effect a pedigree file consisting of 2613 animals was used.

Association analyses were performed using QXPack v.5 (Pérez-Enciso y Misztal 2004, 2011). The nominative p-values for each marker were obtained based on the likelihood ratio tests (LRT). Qxpak5 performs a likelihood ratio test, testing the model with SNP *versus* the model without the SNP, against a chi-square distribution with 1 degree of freedom and this was carried out one SNP at a time

The number of significant markers was chosen based on the False Discovery Rate (FDR) estimated according to the following formula:

$$FDR = \frac{n*P}{k}$$

, where *n* represents the total number of SNPs included in the analysis, *P* is p-value used as a threshold and *k* is the number of markers whose nominal p-value is below the threshold. Markers entering the association analyses were tested for Hardy-Weinberg equilibrium using PLINKv1.07 (Purcel *et al.* 2007). Using the estimate of allele frequencies and assuming the absence of LD (Gianola *et al.* 2013), the percentage of total genetic variance explained by each given marker was estimated as: $2pq\alpha^2 / \sigma^{2.}$. Estimates of additive genetic variances were obtained from a bivariate animal model using the same model showed before but ignoring the effect of the SNPs using TM software package (Legarra *et al*. 2011)

4.- Linkage Disequilibrium windows

The ability to detect any relevant signal leans on the existing LD between the markers and the QTLs. Since the magnitude of the LD varies among populations (Mackay *et al.* 2007) we estimated the LD between the adjacent markers and the LD matrices for all SNP pairs in each chromosome for this population. The estimated LD between markers was used to construct windows around a SNP previously identified as significantly associated with the trait. The genomic regions defined by such windows were then searched to locate candidate genes. The average estimated LD for all markers with respect to adjacent markers was used to establish thresholds in order to construct a window surrounding each significant marker. All markers within the same chromosome showing a LD value above the established threshold were kept, regardless the position of the markers in the chromosome. Then, the searching window was defined by the positions of the two SNPs that yielded a region containing all the other SNPs showing an LD value above the threshold with the significant SNP. Different thresholds were used to determine the searching regions. Estimates of LD were obtained with PLINKv1.07 (Purcel *et al*. 2007). The window was then used to redefine the known QTLs and we assumed that the position of the picks was provided by the significant marker generating the window. Position of picks was obtained in cM by interpolation using the information relative to the the QTLs in cM. The information required was found in www.animalgenome.org.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

1.- Finding significant markers located in known QTLs

Figure 1, shows a Manhattan plot that provides an overall representation of the results found in the association analysis for all the traits IMF, FLA, SF and TEN in both muscles. Chromosome positions are shown in the x-axis and the log of the p-values are represented in the y- axis. Thresholds show on the Figure 1 were set up according to the FDR. The thresholds correspond to a nominal P-values ($p < 0.0001$) to assure an aceptable FDR.

Based on the threshold show in Figure 1, we have been able to find significant SNP markers in Chromosome 1, 5, 10, 14, 15, 16, 25, 29 for the different traits. However, most markers were detected in FD and none of then appeared to be significant in both muscles.

Table 4 and 5 show the number of markers according to the FDR associated to each trait for the FD and PM muscles, respectively. For a FDR ≤ 0.05 only three markers were found to be significant in FD, two for SF and one for TEN.

On the other hand, at the same level of FDR three markers were identified for IMF in PM. However, when we relaxed the criterion (up to 12% for TEN in FD), the number of markers increased up to 15 markers. Thus, as it can be observed in Table 2.1, two more markers were found to be associated with IMF, two associated with FLA, five additional markers associated to SF and finally three more markers were associated with TEN.

We relaxed the FDR to capture more SNPs information associated with the traits that could validate the presence of a QTL in this population.

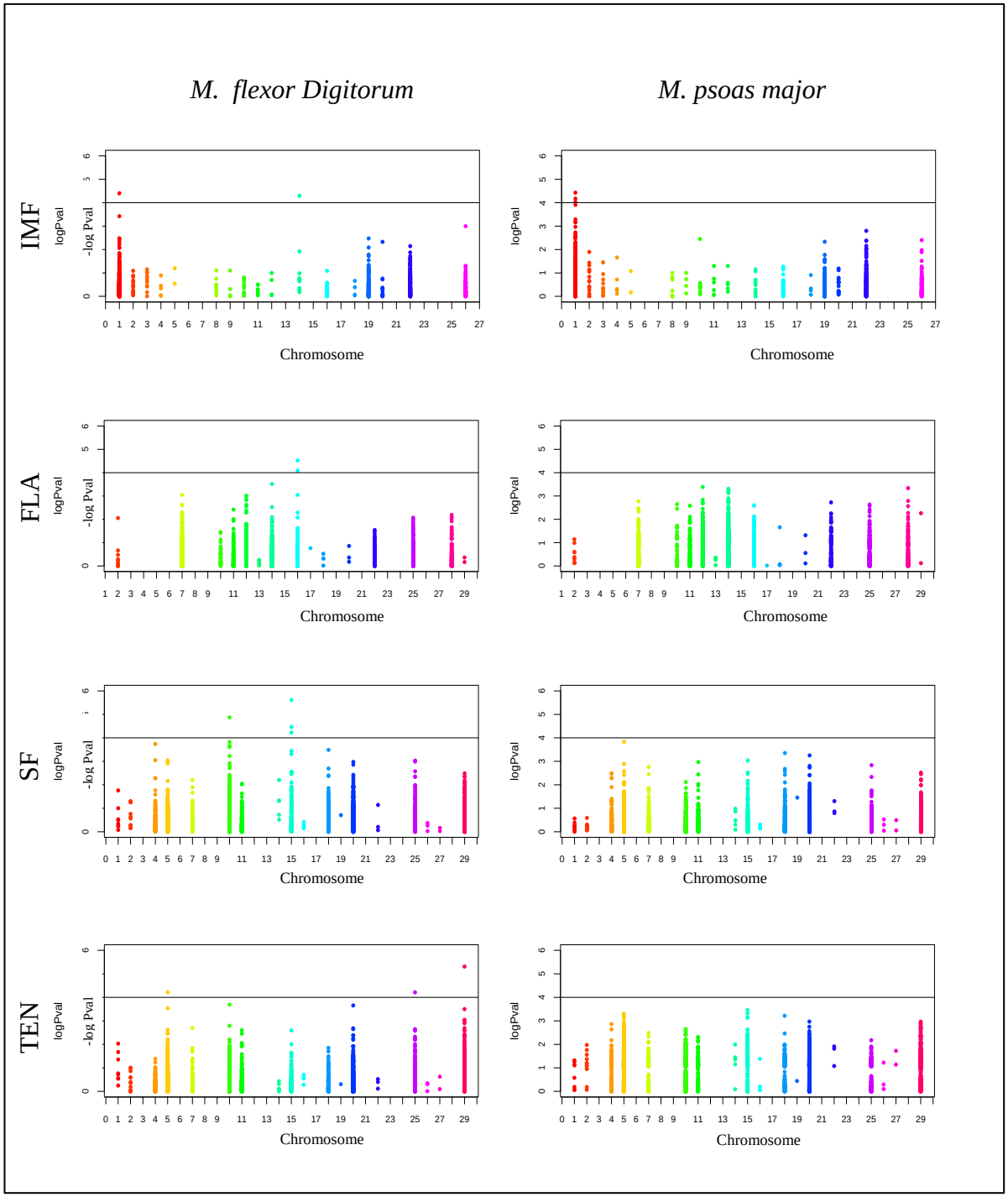

Figure 1. Manhattan plots for Intramuscular fat (IMF), flavor (FLA), shear force (SF) and tenderness, in the *Flexor Digitorum* (FD) and *Psoas Major* (PM). Plots represent the associations between genome-wide single-nucleotide polymorphisms (SNPs) and traits. In all plots, chromosomal positions are in the x-axis and –log (P-values) are in the y-axis.

Table 4. Number of single-nucleotide polymorphisms (SNPs) associated with intramuscular fat (IMF), flavor (FLA), Warner-Blatzer Shear Force (SF) and tenderness (TEN) in the *Flexor digitorum* muscle and, false discovery rate (FDR) according to several P-value thresholds.

P-value	**IMF**		**FLA**		**SF**		**TEN**	
	k	FDR	k	FDR	k	FDR	k	FDR
P<0.01	15	0.75	35	0.64	65	0.53	54	0.64
P<0.001	3	0.38	7	0.32	17	0.2	9	0.38
P<0.0001	2	0.06	2	0.11	5	0.07	3	0.12
P<0.00001	0	0	0	0	2	0.02	1	0.03

Table 5. Number of single-nucleotide polymorphisms (SNPs) associated with intramuscular fat (IMF), flavor (FLA), Warner-Blatzer Shear Force (SF) and tenderness (TEN) in the *Psoas major* muscle and, false discovery rate (FDR) according to several P-value thresholds.

P-value	**IMF**		**FLA**		**SF**		**TEN**	
	k	FDR	k	FDR	k	FDR	k	FDR
P<0.01	46	0.25	84	0.27	43	0.8	153	0.23
P<0.001	7	0.16	7	0.32	4	0.87	8	0.43
P<0.0001	3	0.04	0	0	0	0	0	0
P<0.00001	0	0	0	0	0	0	0	0

In Table 6 and Table 7 are shown the significant SNP markers for each trait as well as their position, their mínimum allele frequency (MAF), the nominal p-value, the effect and the variance explained by the marker for FD and PM, respectvely. Effects for the other muscle are also shown in both tables. For FD (Table 6), markers associated with all traits were found. Thus, for IMF two SNPs were found, one in Chomosome 1 (ARS-BFGL-NGS-43953) and the other in Chromosome 14 (ARS-BFGL-NGS-71749.. Moreover, the one in Cromosome 1, explained arround 21% of the total additive variance and the effect represent arround a 4% of the phenotypic whereas the other one might explianed over 13% of the total additive variance.

Table 6. Effect and P-values (P) of single-nucleotide polymorphisms (SNPs) observed for *Flexor digitorum* muscle (FD), associated with each trait (P < 0.0001), chromosome that are contained (Ch), SNP positions in the chromosome (based on the UMD_3.1 assembly of the bovine genome sequence). Most significant SNPs for each trait in terms of P-values are reported in bold. Minor allele frequency (MAF).

					FD		PM	
Trait	SNP	Chr	[1]Pos. (Mb)	MAF	P	Effect	P	Effect
IMF	**ARS-BFGL-NGS-43953**	**1**	**113.86**	**0.115**	**$3.95x10^{-5}$**	**0.348**	**0.244**	**0.154**
	ARS-BFGL-NGS-71749	14	1.95	0.059	$5.02x10^{-5}$	-0.370	0.696	-0.066
FLA	**ARS-BFGL-NGS-27682**	**16**	**30.19**	**0.204**	**$3.02x10^{-5}$**	**-0.407**	**0.564**	**0.054**
	ARS-BFGL-NGS-18559	16	30.17	0.238	$8.17x10^{-5}$	-0.356	0.416	0.064
SF	**ARS-BFGL-NGS-94765**	**10**	**71.11**	**0.010**	**$3.31x10^{-7}$**	**8.459**	**0.124**	**1.744**
	BTA-105411-no-rs	10	56.76	0.002	$1.34x10^{-5}$	-13.95	0.585	1.665
	ARS-BFGL-NGS-107321	15	33.48	0.243	$6.01x10^{-5}$	-1.713	0.951	-0.021
	BTB-00594438	15	33.57	0.205	$3.42x10^{-5}$	-1.798	0.840	0.051
	BTB-00594449	15	33.60	0.219	$2.46x10^{-6}$	2.004	0.654	0.125
TEN	ARS-BFGL-NGS-10699	5	75.14	0.003	$6.10x10^{-5}$	2.724	0.049	-0.681
	Hapmap48942-BTA-91048	25	32.76	0.351	$6.13x10^{-5}$	-0.387	0.036	0.101
	Hapmap53679-rs29025626	**29**	**45.48**	**0.073**	**$4.89x10^{-6}$**	**-0.738**	**0.063**	**-0.110**

Table 7. Effect and P-values (P) of single-nucleotide polymorphisms (SNPs) observed for *Flexor digitorum* muscle (FD), associated with each trait (P < 0.0001), chromosome that are contained (Ch), SNP positions in the chromosome (based on the UMD_3.1 assembly of the bovine genome sequence). Most significant SNPs for each trait in terms of P-values are reported in bold. Minor allele frequency (MAF).

Trait	SNP	Chr	[1]Pos. (Mb)	[1]MAF	FD		PM	
					P	Effect	P	Effect
IMF	**BTB-01201524**	**1**	**109.36**	**0.482**	**$3.72x10^{-5}$**	**-0.337**	**0.738**	**0.0170**
	ARS-BFGL-NGS-115502	1	109.43	0.498	$6.69x10^{-5}$	-0.324	0.631	0.0240
	Hapmap44213-BTA-122843	1	109.31	0.473	$9.28x10^{-5}$	0.318	0.704	-0.0190

Two SNPs (ARS-BFGL-NGS-27682 and ARS-BFGL-NGS-18559) were identified in chromosome sixteen associated with FLA. The most significant one explained around 16% of the total additive variance and the effect represent arround a 5% of the mean of the trait. In addition, two SNPs (ARS-BFGL-NGS-94765 and BTA-105411-no-rs) that are located in the tenth chromosome and three (ARS-BFGL-NGS-107321, BTB-00594438 and BTB-00594449) in the fifteenth chromosome were associated to SF. The largest effect were found in SNP located in Cromosome 10 whose effect represent more tan 47% of the mean of the traits. However, these results have to be taken with caution as the MAF were extremly low. Finally, we report three markers associated with TEN, the ARS-BFGL-NGS-10699 that is on the fifth chromosome, Hapmap48942-BTA-91048 which is on the twenty-fifth chromosome and Hapmap53679-rs29025626 (FDR= 0.03) is at the twenty-nineth chromosome. The SNP with the lowest p-value was found at Chromosome 29. This particular marker would explain 23.79% of the total additive variance of this trait. Its effect represents an 11% of the phenotipic mean of the trait.

There were not associated markers with FLA, SF or TEN, in PM. However three SNPs were associated to IMF (Table 7). They are BTB-01201524, ARS-BFGL-NGS-115502 and Hapmap44213-BTA-122843 which are located in the first chromosome. Each of those markers should explain arround 30% of the additive genetic variance. Given the overlap between the magnitude of the effects of BTB-01201524 and ARS-BFGL-NGS-115502 (Table 7) and the high LD between them (0.94) (figure 2), it is reasonable to think that both markers are reflecting the same information and are not independent effects, this phenomenon would affect the assignment of variance to a SNP, as described by Gianola *et al.* (2013) where he explains that the standard measures used to assess the contribution of a locus to the variance can be misleading when there is LD and the alleles frequencies are near fixation.

In general, as it has been mentioned the amount of variation explained by the different markers varies from 12.71% (ARS-BFGL-NGS-71749) to 33.36% (BTB-01201524). However, the estimated amount of variance would have correspond to markers in HW equilibtrium, independet of other markers and at intermediate allele frequencies (Gianola *et al.* 2013). All markers were tested to confirm that they were in equilibrium Hardy

Weimberg (HW) with p-value range between $7.5x10^{-3}$ to 1, however, three of them were at extreme low frecuencies and to some extent all of them showed a LD profile with at least other markers in the same cromosome (See Figure 2-5) . With these three factors in mind somehow to assign the percentage of variation to specific markers is missleading (Gianola *et al.* 2013).

At least regarding the set of markers we have included in the analysis muscles appeared to have a differential genetic background. Diaz *et al.* (2006) and López de Maturana *et al.* (unpublised results) found that estimates of genetic correlation for these traits between both muscles were different from 1 and in some cases, as it is the case of IMF, was near cero. On the other hand, markers associated to SF and TEN appeared to be also different in FD though SF is supposed to be an indicator of TEN.

We estimated the genetic correlation between TEN and SF in each muscle. We found that both magnitude and sense changed between them. While the correlation between TEN and SF was negative (-0. 440) in the PM as expected with a high probability of being negative (Pr <0) > 83.04; in FD was positive (0,581) with a high proability of being geater that zero (90.42). Lopez-de Matura *et al.* (2010) using recursive models with this data determined that while in FD, SF was more associated to cologen content; in PM this was related with IMF. SF was obtained in raw meat, because in cook meat the relationship between colágeno and SF is broken (Christiensen *et al.* 2013). In addition, tenderness in FD was evaluated after boiling. According to Purlow (2005) type of cooking, particularly when temperatura is over 56ºC may had a large effect in the intramuscular colagen strength in muscles end therefore in the tenderness (Purslow, 2005). This may be particularly true in muscles with a large content of soluble colagen as it is the case of FD (Torrescano *et al.* 2003).

In this regard, SF on raw meat seems to respond to colagen content of the muscle; however in cooked meat SF might be considered as a mesure of myofibrilar toughness (Torrescano *et al.* 2003; Christiensen *et al.* 2013). Differential temperature at cooking between FD and PM may have altered the relationship between SF and TEN depending upon the muscle. It is known that the greatest changes in connective tissue occur during

meat cooking, influencing the final tenderness of meat (Dubost *et al.* 2013). Above 55ºC and 65ºC respectively, both endomysium and perimysium are denaturated, resulting in a decrease of meat toughness. Moreover, at a cooked meat temperature above 75 °C disintegrate the intramuscular connective tissue breaking the relationship netween connective tissue and meat toughness (Dubost *et al*. 2013).

2.- Using LD information to define QTLs

In the Table 8, we present the average LD between adjacents markers per chromose, the standard deviation (SD), and percentiles 10, 50 and 90. The average estimated LD in the chromosomes of interest, what is to say those where we have identified SNPs associated with traits range from 0.15 in chromosome 25 and 0.189 in chromosome 1.

The average LD found in this population was 0.176. The range of LD across chromosomes ranged betwee 0.141 in chromosome 19 and 0.234 in cromosome 24. Both figures, the ovearll mean and the ranges appear to be lower than the ones reported by Mckay *et al.* (2007) and Lu *et al.* (2012) for a number of cattle populations and crosses. The low LD found in Avilena Negra Ibérica could correspond to a bias because this breed was not included in the original design of the chip, to a low degree of homocigosity in the Avileña Negra-Ibérica breed compare to the others, to a low level of selection in this breed. The reason is subject to research at the moment.

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Table 8. Chromosome number, average LD between adjacents markers and per chromose, the standard deviation (sd), and percentiles 10 (P10), 50 (P50) and 90 (P90). In bold the chromosomes that contains markers associated to traits of interest.

Chromosome	Average	sd	P10	P50	P90
1	**0.189**	**0.271**	**0.002**	**0.063**	**0.624**
2	0.191	0.272	0.002	0.065	0.632
3	0.181	0.258	0.002	0.063	0.587
4	0.171	0.254	0.002	0.056	0.552
5	**0.170**	**0.249**	**0.002**	**0.055**	**0.556**
6	0.191	0.267	0.002	0.066	0.614
7	0.188	0.265	0.002	0.063	0.639
8	0.184	0.266	0.002	0.063	0.629
9	0.184	0.264	0.002	0.058	0.618
10	**0.168**	**0.253**	**0.002**	**0.052**	**0.532**
11	0.175	0.259	0.002	0.054	0.575
12	0.161	0.247	0.001	0.048	0.544
13	0.170	0.255	0.002	0.051	0.536
14	**0.178**	**0.254**	**0.002**	**0.065**	**0.583**
15	**0.166**	**0.248**	**0.002**	**0.054**	**0.509**
16	**0.178**	**0.261**	**0.001**	**0.060**	**0.594**
17	0.166	0.244	0.002	0.054	0.533
18	0.170	0.251	0.002	0.054	0.547
19	0.141	0.223	0.002	0.049	0.420
20	0.163	0.239	0.002	0.060	0.499
21	0.174	0.256	0.002	0.055	0.589
22	0.162	0.242	0.002	0.055	0.475
23	0.198	0.282	0.001	0.051	0.672
24	0.234	0.326	0.001	0.051	0.886
25	**0.150**	**0.225**	**0.001**	**0.051**	**0.452**
26	0.213	0.295	0.001	0.060	0.719
27	0.145	0.221	0.002	0.046	0.431
28	0.143	0.216	0.002	0.047	0.447
29	**0.151**	**0.228**	**0.002**	**0.049**	**0.470**
X	0.158	0.244	0.001	0.047	0.526
Total	0.176	0.257	0.002	0.057	0.576

The average LD found in this population was 0.176. The range of LD across chromosomes ranged betwee 0.141 in chromosome 19 and 0.234 in cromosome 24. Both figures, the ovearll mean and the ranges appear to be lower than the ones reported by Mckay *et al.* (2007) and Lu *et al.* (2012) for a number of cattle populations and crosses. The low LD found in Avilena Negra Ibérica could correspond to a bias because this breed

was not included in the original design of the chip, to a low degree of homocigosity in the Avileña Negra-Ibérica breed compare to the others, to a low level of selection in this breed. The reason is subject to research at the moment.

Based on the magnitude of LD between adajcent markers we decide to establish a threshold of 0.2 to decide the window to search for candidates genes. This figure is over larger tan the average LD found between adjacent markers (Table 8).

In the Table 9, we show the windows created attending to the LD criteria. In this table we include the makers generating the windows or tag markers, what is to say the markers capturing the signal, their chromosome and the position in Mb. In addition we present the markers that are more appart (to the left and right of the tag marker) and in LD over the threshold with the tag markers. This is what we name as a window or QTLs region where to search for candidate genes. In the case of ARS-BFGL-NGS-71749 and Hapmap53679-rs29025626 the initial position of the window is established by these two markers since there are no markers to the left in sufficient LD to delimiter the window. In the opposite side, markers ARS-BFGL-NGS-94765 and ARS-BFGL-NGS-18559 represent the end of the window because there are not markers to the right in LD.

Table 9. Summary of the LD windows generated by significantly associated markers identified for the traits analysed. These LD windows were generated considering a Threshol LD> 0.2. The markers represent the initial positions LD window are those which are above the threshold and are to the right away with respect to significant marker, markers representing the final position of LD winsows are those which are above the threshold and are to the left. In some cases the beginning or end of the LD window is delimited by the own marker significant, since no markers underlying are above the threshold.

Marker identified[1]	Ch[2]	Position[3]	Marker IPLD[1]	IPLD[3]	Marker FPLD[1]	FPLD[3]	LDw[4]
ARS-BFGL-NGS-43953	1	113.86	BTB-01058479	113.67	ARS-BFGL-NGS-46265	114.38	0.71
Hapmap44213-BTA-122843	1	109.31	Hapmap49986-BTA-41525	90.80	ARS-BFGL-NGS-115502	109.43	18.63
BTB-01201524	1	109.36	BTB-01631727	100.80	ARS-BFGL-NGS-115502	109.43	8.63
ARS-BFGL-NGS-115502	1	109.43	BTB-01631727	100.80	BTB-01575226	116.52	15.72
ARS-BFGL-NGS-10699	5	75.14	ARS-BFGL-NGS-100634	57.34	BTA-74507-no-rs	86.46	29.13
BTA-105411-no-rs	10	56.76	BTB-00424791	52.63	ARS-BFGL-NGS-94765	71.11	18.48
ARS-BFGL-NGS-94765	10	71.11	BTA-105411-no-rs	56.76	ARS-BFGL-NGS-94765	71.11	14.35
ARS-BFGL-NGS-71749	14	1.95	ARS-BFGL-NGS-71749	1.95	BTA-35941-no-rs	2.28	0.32
BTB-00594438	15	33.57	ARS-BFGL-NGS-107321	33.48	ARS-BFGL-NGS-106172	34.32	0.84
BTB-00594449	15	33.60	ARS-BFGL-NGS-57210	32.64	Hapmap57840-rs29014510	34.34	1.70
ARS-BFGL-NGS-107321	15	33.48	ARS-BFGL-NGS-106188	31.55	Hapmap36832-SCAFFOLD135185_8729	38.54	6.99
ARS-BFGL-NGS-18559	16	30.17	Hapmap53163-rs29012001	30.09	ARS-BFGL-NGS-27682	30.19	0.10
ARS-BFGL-NGS-27682	16	30.19	ARS-BFGL-NGS-89576	29.89	ARS-BFGL-NGS-27682	30.17	0.28
Hapmap48942-BTA-91048	25	32.76	ARS-BFGL-NGS-81014	30.55	ARS-BFGL-NGS-74596	35.78	5.23
Hapmap53679-rs29025626	29	45.48	Hapmap53679-rs29025626	45.53	ARS-BFGL-NGS-35555	45.58	0.05

[1] Marker identified: Name of the Marker significative associated to traits analysed; Marker IPLD: Name of marker in the Initial position of LD window; Marker FPLD: Name of marker in the Final position of LD window.[3]Position: Position in the chromosome of marker identified.
[2] Chromosome
[3] Position: Position in the chromosome in Megabases (Mb) of the significative marker; IPLD: Initial position of LD window (Mb); FPLD:Final position of LDwindow (Mb).
[4] Size in Megabases (Mb) of LD Windows generate

In Table 10 we show the validated QTLs for IMF, FLA, SF and TEN as well as the for this population and average size windows generated for the markers with LD > 0.20 of the SNP associated to each trait. In addition we show the position of the peak of the published QTL as well as the "new peak" or the position of the tag marker in cM.

The SNP identified in the FD and the three SNPs identified in the PM are all located at chromosome 1 and significantly associated with IMF. All of then are cointained in the in the QTL reported by Gutiérrez-Gil *et al*. (2008) with a size of 26.37 Mb. These SNPs validated the presence of an important region for this trait, these markers generate a window LD whose average size is 10.922 Mb. This would represent a reduction of 58.58% of the size of published QTL. We observe the opposite with the SNP associated to IMF in Chromosome 14. The QTL ID 12029 reported by Thaller *et al*. (2003) measures 0.153 Mb but the LD window generated measures 0.322 Mb, incrementing 110% the size vs QTL size. In any case, the size of the QTL is small as compared to others.

For FLA, the SNPs asociated are located within the QTL ID 4837. This measures on average 13.256 Mb, however the size of the window generated by the sifnificant markers measure an average of 0.711 Mb. This represents a reduction in almost 95% the size of the published QTL.These SNPs validated the presence of a región asociated to FLA.

Two SNPs in the teenth chromosome were associated to SF, these SNPs validated the presence of the QTL whose ID is 4826. The LD windows generated presents an average size of 16.412 Mb. This figure represents a reduction in almost 19% compared with the QTL size repored in this region. In the fifteenth chromosome we could also validate the QTL ID 4836 report that identified three SNPs whose LD windows average 3.179 Mb. This represents a reduction in 69.17% compared to QTL size. Finally, three markers associated with TEN, validated the presence of six QTLs regions for this trait. One SNP identified in the fifth chromosome refers to a QTL ID 1365 that measures 49.770 Mb and have been reported by Casas *et al.* (2000), the LD window generated for the SNP reduced in 41.47% the size of the published QTL. In the twenty-fifth chromosome we found SNP that could validate the QTL ID 4848 reported by Gutiérrez-Gil *et al.* (2008), and the LD windows generated for the SNP reduce in 49.22% the size of the published QTL.

Moreover the SNP identifies in the twenty-nineth chromosome was cointained in several QTLs such as QTLs ID 4852, 4853 reported by Gutiérrez-Gil *et al.* (2008), the ID QTLs 1373, 1374 reported by Casas *et al.* (2000) and QTL ID 1345 reported by Casas et al., (2003). The size of the LD windows generated by this SNP cause a reduction in 99.25% compared to the average size of published QTLs.

Table 10 Size of published QTLs as well as the location and ID for the traits studied as well as the average LD windows and changes in size of the searching window relative to QTLs. QTL peak position and SNP position. Trait, Chromosome with markers associated (Chr) and peak of (ID QTL).

Trait	Chr	Nº SNPs	ID QTL	[1]Size QTL	[2]Size of window LD	[3]% Changes in size	[4]QTL Peak	[5]SNP position
IMF	1	3	4819	26.37	10.92	58.58	115.70	105.57
	14	1	12029	0.15	0.32	110.37	0.50	--
FLA	16	2	4837	13.25	0.71	94.63	39.10	45.10
SF	10	2	4826	20.23	16.41	18.90	66.40	68.87
	15	3	4836	10.31	3.17	69.17	42.90	33.28
TEN	5	1	1365	49.77	29.12	41.47	66.00	149.48
	25	1	4848	10.29	5.22	49.22	50.50	40.12
	29	1	4853	17.16	0.09	99.98	56.30	54.74
	29	1	4852	17.16	0.09	99.98	52.10	54.74
	29	1	1374	14.17	0.09	99.99	65.00	63.49
	29	1	1373	9.97	0.09	99.99	56.00	61.46
	29	1	1345	20.66	0.09	99.98	54.00	51.40

[1] Size in Mb for all QTL including SNP identified
[2] Mean in Mb of size for all LD windows generated for all SNP identified
[3] Changes of windows size relative to published QTL in percentages. Values greater than 100 means increases in size
[4] QTL peak location in cM
[5] SNP position associted in cM (calculed for interpolación of Mb to cM in each QTL)
-- Can not be calculated

In this study, we have validated twelve QTLs reported in relation to several meat quality traits. This is an interesting result particularly because they have been validated in the Avileña Negra-Ibérica breed which is one of the Spanish local breed quite different from the breeds or crosses where most of published QTLs have been found. These results provide some support for the success of multibreed genomic selection for these type of

traits. In chromosome 5 the distance between the QTL 1365 and the SNP ARS-BFGL-NGS-10699 was 83.48 cM. This suggests the existence of a new candidate position in this same QTL region associated to TEN. In all other cases the distance between the QTL peak and the associated SNP does not differs more than 10 cM. Thus, the minimum distance between the QTL peak and the associated SNP was 1.51 cM in chromosome 29 and the maximum distance between the QTL peak and the associated SNP was 10.38 cM in chromosome 25.

3.- Describing what is in the QTLs

In order to gain some insight about the genetic background of traits, in Figure 2-4 we present the LD profiles between significant markers and the markers in the same chromosome. Markers that are represented are those above a LD value ranging between (0.02 and 1). Values depend upon each chromosome. Figures 2-4 are presented by trait. To gain knowlegde about the regions, we developed windows to search base on the results of the analysis and the estructure of LD. Continuous lines in paralel to Chromosome positions in different colours represent the window generated for each marker. In green we always represent the extent and position of the published QTLs. As it has been said, to establish the window we use markers in an LD above 0.2 with the significant markers.

QTLs for Intramuscular Fat

As it is shown in Figure 2, we found a region between the initial position of QTL ID 4819 and the final position of the LD window generated for the ARS-BFGL-NGS-115502 markerwhich is located betwen 106.9-116.52 Mb, This region is an overlapping area of all LD windows for all significant markers associated to IMF in the first chromosome. This LD window contains 130 genes. We performed an enrichment analysis (DAVID: Database for Annotation, Visualization, and Integrated Discovery; Dennis *et al.* 2003) with these genes and we found reported of which are mentioned nine in biological processes (IL12A, PLD1, PTX3, PRKCI, P2RY1, GHSR, LOC784297, CLDN11, NLGN1), five in molecular functions (GPX5, GPX6, PLD1, PRKCI, LOC784297) and seventeen in cellular

components (LOC507471, SEC62, NCEH1, B3GALNT1, CLDN11, GHSR, C3orf57, KPNA4, MFSD1, NLGN1, KCNAB1, PPM1L, P2RY1, SSR3, LOC527195, SLC2A2, SLC7A14).

In Figure 2 we can also observe the window generated by marker associated to IMF in FD found in Chromosome 14. The published QTL was described by Thaller *et al.* (2003) as associated to IMF in in Longissimus dorsi and semitendinoso muscles. The enrrichmen analysis highlight two genes MAPK15 and NRBP2 that genes are involved in biological processes of lipids and both including PUF69 gene that is also described in molecular processes of lipids.

Thaller *et al.* (2003) identified as a candidate gene the DGAT1 that is a gene that is directly related to the synthesis of triglycerides (Harris *et al.* 2011). That position of the QTL in the asemble UMD_3.1, does not contain DGAT1 gene and the higher LD with adjacent markers appears in the opposite direction to DGAT1. However, as it can be observed in Figure 2, there is a peak of LD near the threshold with a marker that is contained in the DGAT1 gene. That specidic was not included in the analysis because under the UMD3.1 asemble that marker was not located in the published QTLs. In additon to this QTL, several authors have described mutations in DGAT1 associated to IMF (Thaller *et al.* 2003; Casas *et al.* 2005). Therefore, it could be interesting for futures studies to search for mutations in the set of genes previously mentioned as well as the DGAT1 in the ANI population.

However, as well as the significant marker (see Table 6), markers contained in the 50K chip in this region apparently are fixed in this poblation, what may suggest that it is a region that has been subject to some sort of selection proccess. Reciently, chromosome 14 has been identified as a chromosome rich in RHO (regions of homocigosity) in cattle (Deidre *et al.* 2012) which somehow provides support to the hypothesis previously stated. It is known that DGAT1 has been associated to the synthesis of tryglcerides which are essential in all mammals species to provide structure to cells (Harris *et al.* 2011) and therefore it could be reasonable to observe a lack of variability to assure the necessary formation of such compounds.

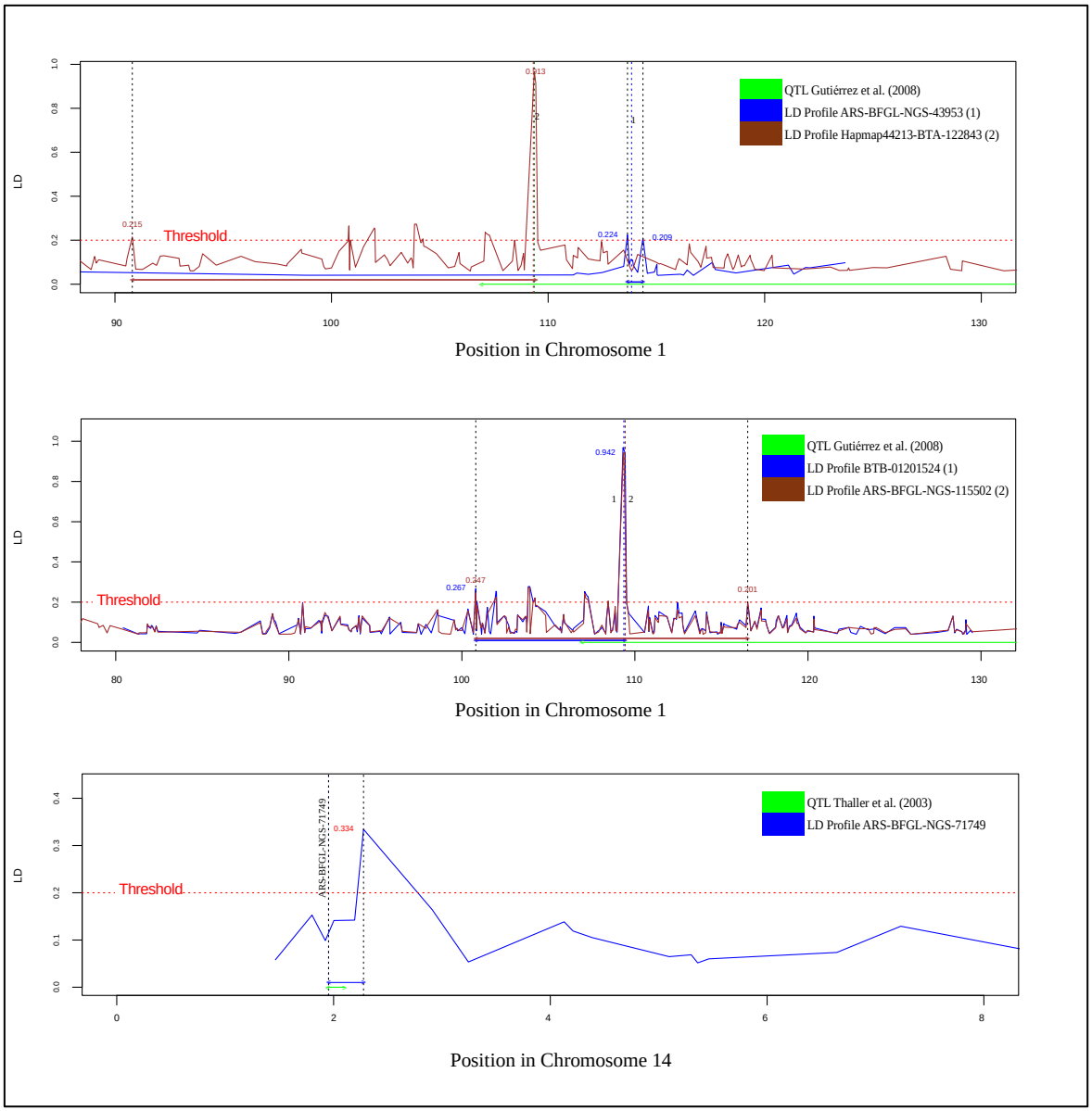

Figure 2. Linkage Disequilibrium (LD) profiles of markers associated to intramuscular fat (IMF) in Chomosome 1 and 14 for both muscle (FD and PM). In the x-axis LD values ranging from 0-1 calculated with chi square (Plink software). The x-axis is scaled in millions of base pairs (Mb). Lines parallel to the y-axis: blue or brown dot line indicates postion of markers identified; black dot line indicates a window formed between the significant marker and the most distant markers in a LD above the threshold. Lines parallel to the x-axis: red dot line indicates the threshold LD (0.2); green line indicates the length of the QTL published; blue or brown lines indicates the window LD length generated corresponding to the color markers.

QTLs for flavor

In the case of FLA the search windows generated by the two associated SNPs are almost completely overlapping.

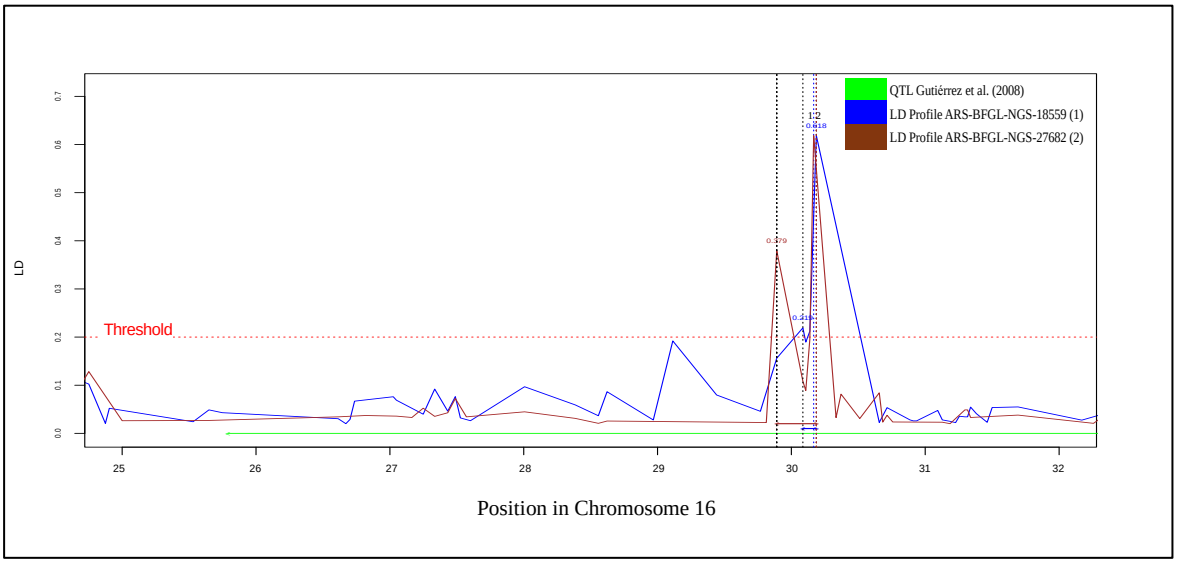

Figure 3. Linkage Disequilibrium (LD) profiles of markers associated to flavor (FLA) in Chomosome 16 for *Flexor digitorum* muscle. In the x-axis LD values ranging from 0-1 calculated with chi square (plink software). The x-axis is scaled in millions of base pairs (Mb). Lines parallel to the y-axis: blue or brown dot line indicates postion of markers identified; black dot line indicates a window formed between the significant marker and the most distant markers in a LD above the threshold. Lines parallel to the x-axis: red dot line indicates the threshold LD (0.2); green line indicates the length of the QTL published; blue or brown lines indicates the window LD length generated corresponding to the color markers.

In the window generated by the SNP there are twenty genes which to our knowledge have not been previously associated to this trait. However, some of these genes have been found in relation to the formation of fatty acids that are responsible for the palatability of meat (Faucitano *et al.* 2008**)**, One of these genes is ACBD3 that encodes a protein inhibitor of SREBP1 promoter activity which is a transcription factor involved in the regulation of lipogenesis through the action if insulin . Thus, ACBD3 plays an essential role in maintaining lipid homeostasis by regulating SREBP1 processing and thus affect cellular lipogenesis Chen *et al.* (2012), SREBP-1 can down regulate gene expression when a

deprivation of food is applied and the level of insulin is low, while on the other hand it can enhance gene expression when rodents are placed on a high carbohydrate diet. In contrast with glucose and insulin, when a diet rich in polyunsaturated fatty acids is fed, a down regulation of lipogenic gene expression is observed *via* the recruitment of SREBP-1 (Laliotis *et al.* 2010).

QTLs for Warner-Blatzer Shear Force

In Figure 4 can be seen on chromosome 10 and the position the two SNP associated SF, the search window generated by the SNP BTA-105 411-no-rs is a region that containing over fourteen Mb bases, here overlap the QTL and LD windows of both markers. This QTL region was reduced by LD windows generated is large and contains about 121 genes described among which can be found genes involved in processes for energy production (GALK2, MAPK6, MYO5A, MYO5C, ATP8B4, SPATA5L1, TRPM7), among others. Thus, it is an interesting region to further search for genes whose functions are interfering with the expression of this character. However, as it has been menyiones, results for this particular chromosome should be taken cautiously because MAF are extremely low.

In Figure 4, fifteen-chromosome show the search windows generated by the three SNPs identified associated to SF. The BTB-00594438 SNP generated a search window which comprises the region (33.48 - 34.32 Mb) (table2.6) where overlapping windows generated by the other two markers and the QTL, LD in this small window there are nine genes described in NCBI for cattle (LOC100848689, UBASH3B, CRTAM, C15H11orf63, LOC100337073, BSX, LOC781922, HSPA8, CLMP) among which we highlight HSPA(Hsp70) linked to cellular apoposis (Hoquette et al., 2012).

BSX gene also there, interesting because has been studied in mouse where it was determined their involvement in growth control, Tara and Akihira (2007), but still no function described in cattle.

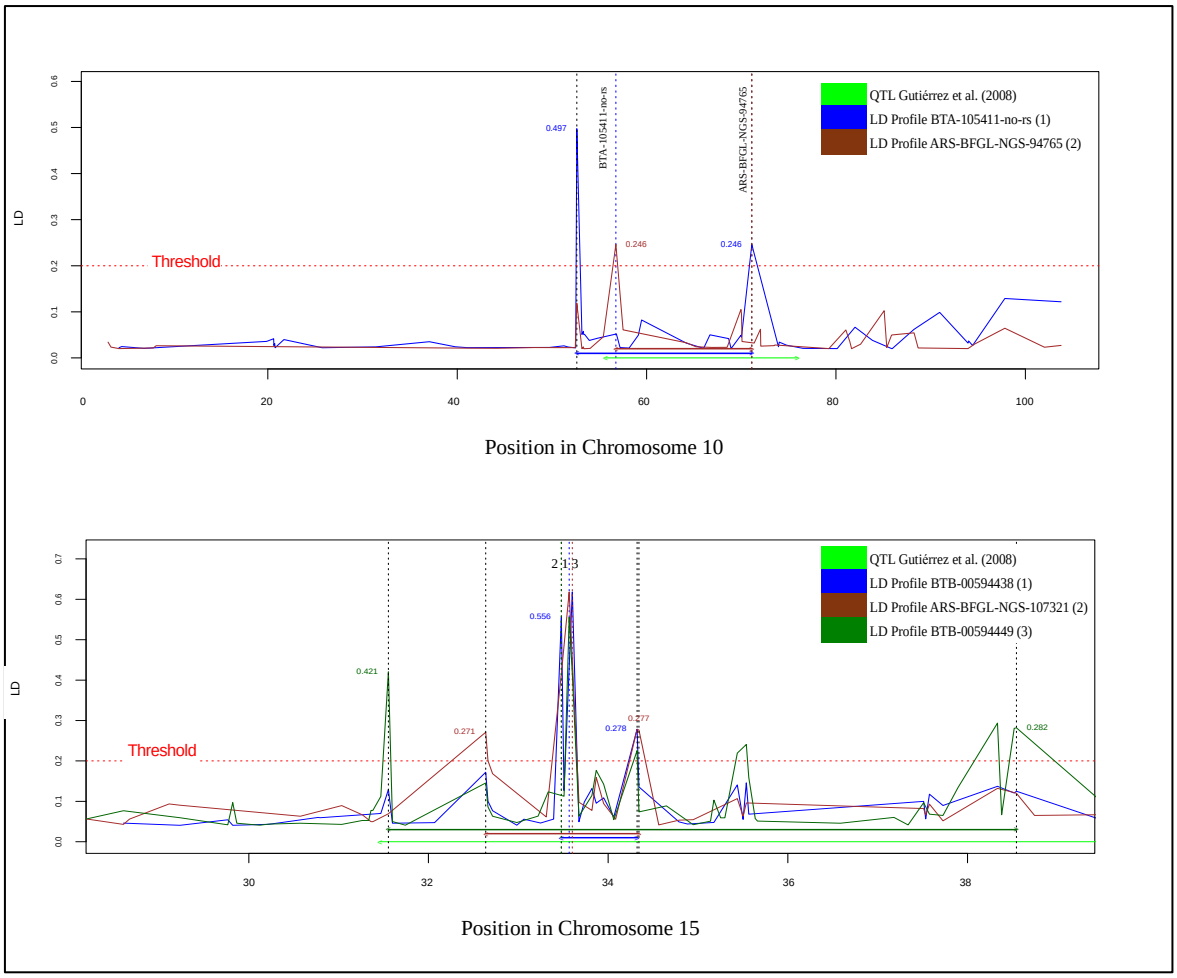

Figure 4. Linkage Disequilibrium (LD) profiles of markers associated to shear force (SF) in Chomosome 10 and 15 for *Flexor digitorum* muscle. In the x-axis LD values ranging from 0-1 calculated with chi square (software plink). The x-axis is scaled in millions of base pairs (Mb). Lines parallel to the y-axis: blue, brown or dark green dot line indicates postion of markers identified; black dot line indicates a window formed between the significant marker and the most distant markers in a LD above the threshold . Lines parallel to the x-axis: red dot line indicates the threshold LD (0.2); green line indicates the length of the QTL published; blue, brown or dark green lines indicates the window LD length generated corresponding to the color markers.

A peculiar data was that the SNP ARS-BFGL-NGS-107321 and BTB-00594438 have a high linkage disequilibrium with each other and show effects of similar magnitude (Table 4) may indicate that both reflect the same information, the contrary caseSNPs observed

with BTB-00594449 which is in the same region and has a high linkage disequilibrium (0.4 approx.) with respect to the other two SNP, but the magnitude of the effect is different.

QTLs for Tenderness

For TEN trait the search window generated on chromosome five is oversized with more than twenty-nine million base pairs (Figure 5) and approximately three hundred eighty-one genes, that given the magnitude the effect on the character and the percentage of variance explained by this SNP (Table 5), would be convenient tp make an exhaustive in an exhaustive search of candidate genes. Among all of this, we have identified two chaperores DNAJC14 tenderness (Hoqcette *et al.* 2012) and HSP90B1 (Picard *et al.* 2013) that in previous studies have been associated with tenderness via mechanisms of celular apoptosis.

In the Figure 5 it is shown the LD profile corresponding to Hapmap48942-BTA-91048 at twenty-fifth chromosome. This marker has generated a searching window of approximately 5.23 Mb (Table 9). There is an overlap of more than 30% of the QTL area, in a region with one hundred thirty two reported genes. In this region we have found HSPB1 that is a short chaperon that has been also previouly reported in assocition to tenederness and intramuscular fat content (Kim *et al.* 2011; Hoqcette *et al.* 2012) and Picard *et al.* 2013). In addition to this gene, there are two of cytochromos (COX6A2, CYP3A4) which are also involved in the oxidative metabolims and therefore with a potential influence on tenderness.

In Figure 6 also shows the searching window that generated the SNP identified on chromosome twenty-nineth. All there QTLs have been described in association to CAPN1 Casas et al. (2000; 2007). This gene is at a distance of 1.368 cM to the left of the SNP associated to tenderness (Hapmap53679- rs29025626). When we observe the LD profile of this marker with SNP located toward the direction of CAPN1, this LD tends to zero. Therefore, it suggests that we should look for genes located in the other direction. In this

direction there are five genes that need to be investigated in relation to their effect on TEN.

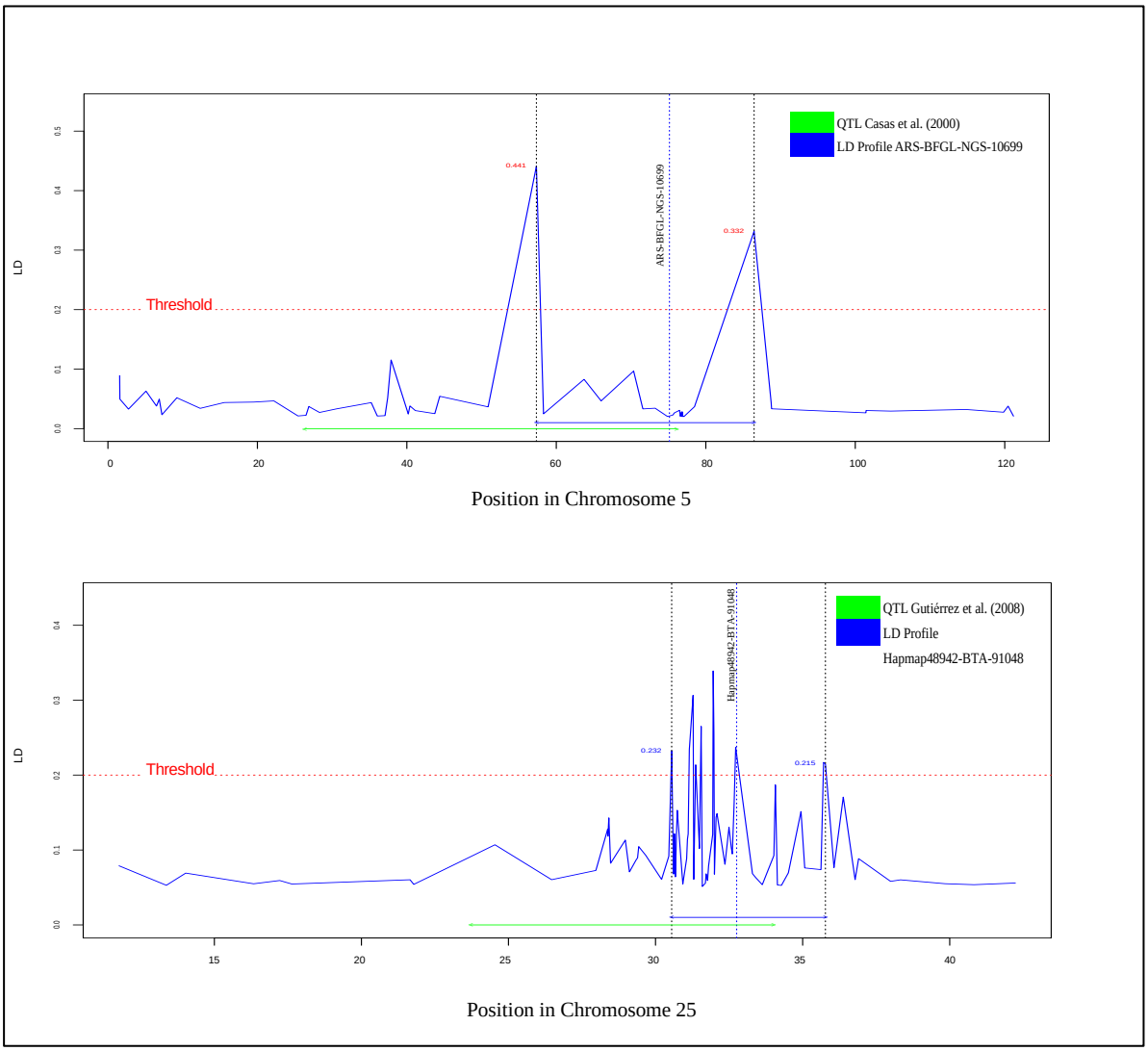

Figure 5. Linkage Disequilibrium (LD) profiles of markers associated to tenderness (TEN) in Chomosome 5 and 25 for Flexor digitorum muscle. In the x-axis LD values ranging from 0-1 calculated with chi square (plink software). The x-axis is scaled in millions of base pairs (Mb). Lines parallel to the y-axis: blue dot line indicates postion of markers identified; black dot line indicates a window formed between the significant marker and the most distant markers in a LD above the threshold. Lines parallel to the x-axis: red dot line indicates the threshold LD (0.2); green, brown, purple and pink lines indicates the length of the QTLs published; blue line indicates the LD window length generated by the markers.

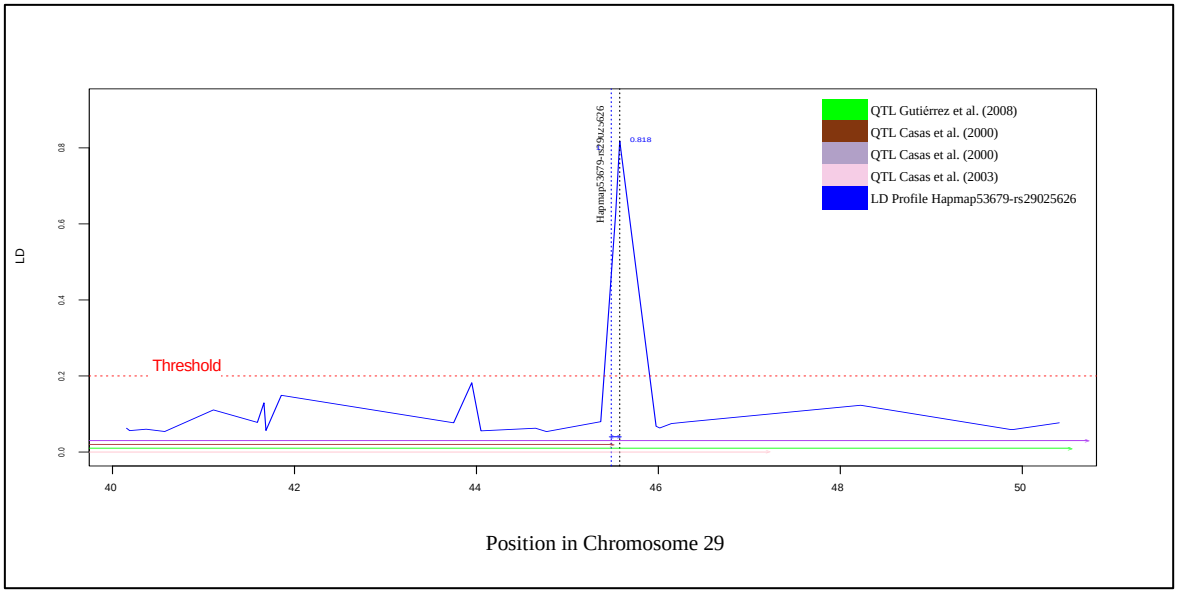

Figure 6. Linkage Disequilibrium (LD) profiles of markers associated to tenderness (TEN) in Chomosome 29 for Flexor digitorum muscle. In the x-axis LD values ranging from 0-1 calculated with chi square (plink software). The x-axis is scaled in millions of base pairs (Mb). Lines parallel to the y-axis: blue dot line indicates postion of markers identified; black dot line indicates a window formed between the significant marker and the most distant markers in a LD above the threshold. Lines parallel to the x-axis: red dot line indicates the threshold LD (0.2); green, brown, purple and pink lines indicates the length of the QTLs published; blue line indicates the LD window length generated by the markers.

As we can observe in Figures 2 to 6, in most cases the use of the LD information has allowed us reducing the size of searching window in comparison to the published QTLs (Table 10), with the exception of the LD window generated by ARS-BFGL-NGS-71749 which was slightly larger than the size of the QTL published.

In many ocassions, one of the constraints of association analyses is the size of the experiments that does not allow very conclussive results. Therefore, validation of results across populations maybe an indicator of the validity of results.

FINAL CONCLUSIONS

FINAL CONCLUSIONS

1. We have validated a number of QTLs that have been previously described in another type of populations however, provided the low frequencies for some of the markers we need to be cautious with some of the results, particularly those affecting Chromosome 10.

2. As it has been presented here, the existing LD profiles may help to redefine the QTLs using SNP information. In order to do this, factors affecting the estimate of LD should be carefully analyzed.

3. Results suggest differences in the genomic regions affecting IMF, FLA, SF and TEN between muscles.

4. We have observed that the highest peaks of LD are not always present among adjacent markers. Weather or not this is an indication of errors in position of markers or just the effect of other genetic mechanisms is something that needs to be elucidated.

5. We have identified a number of candidate genes related to IMF, FLA, SF and TEN as well as we have proposed a number of them. It is of particular interest the region found in chromosome 29 associated to TEN. Taking into consideration the LD in this population, the causal mutation will take us apart from CAPN1 gene. This establish the hypothesis of a new existing QTL region related to TEN in the ANI population.

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