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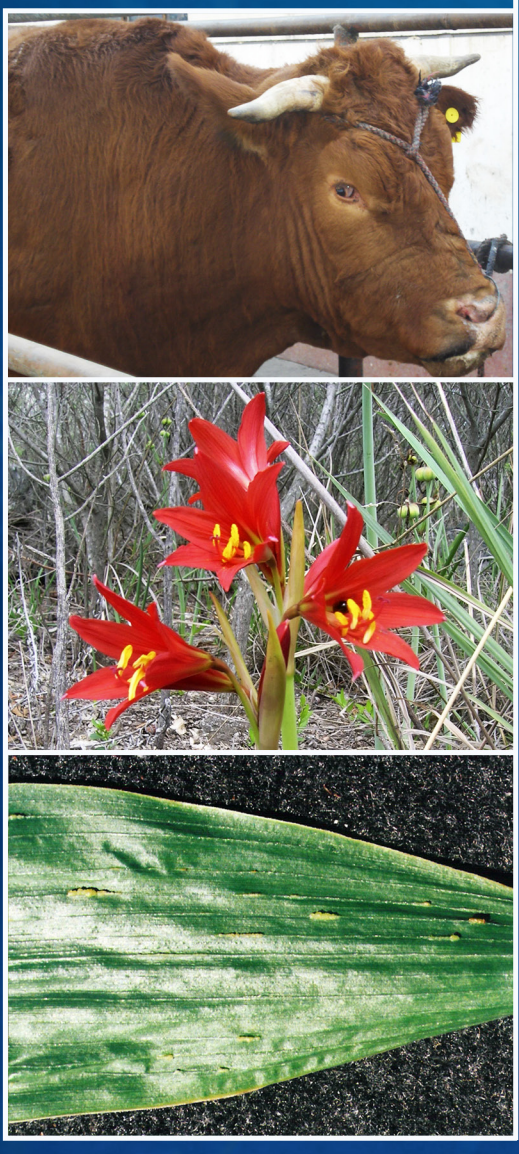


Cited by
BIOLOGICAL ABSTRACTS
GENETICS ABSTRACTS
SISTEMA LATINDEX

Included in **SciELO**

BUENOS AIRES - ARGENTINA

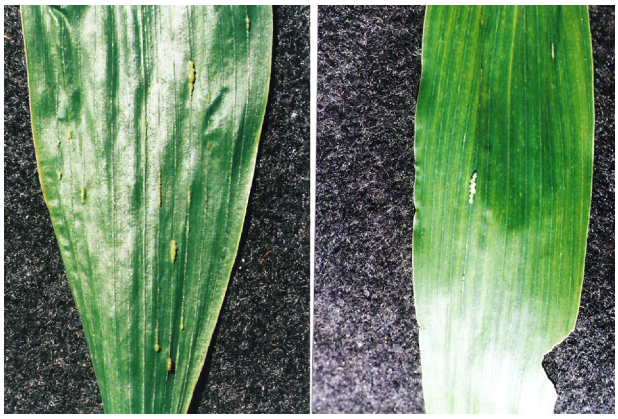
COMITÉ EDITORIAL

ÍNDICE

FOTOGRAFÍAS Y AUTORES

Tapa

Evaluation of six single nucleotide polymorphisms for bovine traceability in the context of argentine-chinese beef trade.
Autor: M. V. Ripoli - G. Giovambattista



Mapeo de QTL para una medida multivariada de la reacción al virus del mal de Río Cuarto.
Autor: N.C. Bonámico

Rodophiala bifida ("azucenita roja", endémica de Argentina y Uruguay).
Autor: M.L. Echeverría

LA ESTRUCTURA GENÉTICA DE POBLACIONES DE PLANTAS CONDICIONA LA INTERPRETACIÓN DE PARÁMETROS Y SU ALCANCE EN CARACTERES ECOFISIOLÓGICOS

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ABSTRACT

It is almost a constant in the ecophysiology papers that, explicitly or implicitly, the genetic structures of the studied material were clearly present as sources of tolerance (germplasm), contrasting materials (genotypes) or simply as cultivars. The "genetic structure" of a population refers to the type, amount and distribution of genetic variation present in the population and it is expressed in terms of gene or genotype frequencies. There are five types of cultivars (populations, synthetic, pure lines, hybrids and clones) considering that all commercial varieties fit into any of these five types or their variants. The objective of this work was to analyze the ecophysiological characters studied in the major crops and their scope and limitations in relation to the genetic structure of populations. An ecophysiologist who studies physiological characters or interactions of the crop with environmental factors, should bear in mind these genetic structures when choosing materials (morphological types, cultivars or genotypes) and when he/she reaches his/her conclusions and makes recommendations, since they will determine the scope of the study. Likewise, the contributions of ecophysiology to describe classify and explain how plants perform in a particular situation and with a given genetic structure are analyzed.

Key words: genetic structure, ecophysiological characters, plant populations, cultivated varieties

RESUMEN

Es casi una constante en los trabajos de ecofisiología que, explícitamente o implícitamente, las estructuras genéticas del material en estudio estén claramente presentes, como fuentes de tolerancia (germoplasma), como materiales contrastantes (genotipos) o simplemente como cultivares. Se entiende por "estructura genética" de una población al tipo, cantidad y distribución de la variación genética presente en la misma y se expresa en términos de frecuencias génicas o genotípicas. En este trabajo se exponen los cinco tipos de cultivares (poblaciones, sintéticos, líneas, híbridos y clones) ya que todas las variedades comerciales encajan en alguno de los cinco tipos o sus variantes. El objetivo de este trabajo fue analizar en el estudio de los caracteres ecofisiológicos de los principales cultivos, los alcances y las limitaciones en relación a la estructura genética de las poblaciones cuando la interpretación de resultados será aplicada en el manejo del cultivo o en el mejoramiento del mismo. Un ecofisiólogo que estudia caracteres fisiológicos o interacciones del cultivo con factores ambientales, debería tener presente esas estructuras genéticas en el momento que elige los materiales de estudio (tipos morfológicos, cultivares o genotipos) y cuando obtiene sus conclusiones y efectúa recomendaciones, ya que ambos determinarán los alcances de su estudio. Asimismo se analizaron los aportes de la ecofisiología para describir, clasificar y explicar cómo se comportan las plantas de un cultivo en una situación particular y con una estructura genética dada.

Palabras clave: estructura genética, caracteres ecofisiológicos, poblaciones de plantas, variedades cultivadas

INTRODUCCIÓN

La bibliografía existente sobre el estudio de caracteres ecofisiológicos de los principales cultivos es muy vasta, variable en muchos aspectos aunque con un cúmulo de trabajos que responden, para determinados caracteres, a un modelo de análisis sólido desde lo fisiológico, pero con alcances de las conclusiones no siempre acordes a la estructura genética del material estudiado. Es casi una constante en esos trabajos, que explícitamente o implícitamente, las estructuras genéticas del material en estudio estén claramente presentes, como fuentes de tolerancia (germoplasma), como materiales contrastantes (genotipos) o simplemente como variedades o cultivares (poblaciones, líneas, clones o híbridos). Aunque esas estructuras genéticas estén presentes no siempre son consideradas al elegir los materiales, extraer conclusiones, o hacer las recomendaciones de los trabajos que centran los estudios en caracteres ecofisiológicos. Esas estructuras también están presentes en poblaciones naturales de diferentes ecosistemas con bibliografía abundante que evalúa las estructuras poblacionales, con caracteres fisiológicos vinculados a procesos evolutivos, de filogenia y de comunidades en diferentes hábitats.

Para relacionar caracteres ecofisiológicos con el fitomejoramiento, se requiere poner énfasis en el análisis de la estructura genética de la población en estudio con el propósito de determinar los alcances y las aplicaciones agronómicas. Para definir el alcance en la utilización agronómica de los resultados de ensayos con esos caracteres, también se deberían tener siempre presentes a la estructura genética y a la fuente de variabilidad de los materiales probados. El objetivo de este trabajo fue analizar en el estudio de los caracteres ecofisiológicos de los principales cultivos, los alcances y las limitaciones en relación a la estructura genética de las poblaciones cuando la interpretación de resultados será aplicado en el manejo del cultivo o en el mejoramiento del mismo, haciendo mención también a estudios de poblaciones nativas.

MARCO DE REFERENCIA

El análisis de la bibliografía sobre ecofisiología se circunscribió a lo estrictamente necesario para desarrollar este trabajo de opinión acorde al objetivo. La inclusión limitada de bibliografía no tuvo otra finalidad que ilustrar cómo los caracteres ecofisiológicos están relacionados con estructuras genéticas particulares. Tanto los trabajos citados como los que no han sido citados y responden a un mismo patrón, merecen el mismo comentario desde la genética sin que ello signifique una objeción o una aprobación tácita. Lo aquí expresado se debe considerar sólo como una reflexión general en la que se apunta a la estructura genética del material estudiado, más que al trabajo particular elegido para el ejemplo.

Para mejor comprensión de las estructuras genéticas de los cultivares, en los siguientes párrafos se desarrollarán sintéticamente los conceptos de *creación fitogenética y variedad* (INASE) y los diferentes *tipos de cultivares*, conforme a la Ley de Semillas de la Argentina y a la bibliografía más específica.

Creación fitogenética: Toda variedad o cultivar, cualquiera sea su naturaleza genética, obtenido por descubrimiento o por incorporación y/o aplicación de conocimientos científicos.

Variedad: Conjunto de plantas de un solo taxón botánico, del rango botánico más bajo conocido, que pueda definirse por la expresión de los caracteres resultantes de un cierto genotipo o de una cierta combinación de genotipos y pueda distinguirse de cualquier otro conjunto de plantas por la expresión de uno de dichos caracteres, por lo menos. Una variedad en particular puede estar representada por varias plantas, una sola planta o una o varias partes de una planta, siempre que dicha parte o partes puedan ser usadas para la producción de plantas completas de la variedad.

Tipo de cultivares: En la Tabla 1 se muestran los cinco grandes grupos de cultivares, ya que todas las variedades comerciales se ajustan a alguno de ellos.

Tabla 1. Los cinco grandes tipos de variedades cultivadas (cultivares) (Gallais, 1990)

Tipo de cultivar	Descripción
Variedad Población	Ecotipos o poblaciones artificiales por selección
Variedad Sintética	Poblaciones artificiales de *n* genotipos en alógamas
Variedad Híbrido	F1 de cruzamiento controlado de líneas o clones o familias
Variedad Línea Pura	Línea fijada (un solo genotipo)
Variedad Clon	Un solo genotipo de multiplicación vegetativa o apomíctica

Algunas variantes como las variedades Multilíneas son una derivación de las líneas puras con más de un genotipo homocigota para otorgarle plasticidad o tolerancia a distintas razas de patógenos, entre otros. Mientras que los Semihíbridos son variantes de los Híbridos en las que los cruzamientos no son totalmente controlados y se asemejan a una variedad Sintética. Una variante similar a las Multilíneas pueden encontrase en variedades Multiclones.

Para la comprensión de las estructuras genéticas de especies nativas es necesario remitirse a Genética de Poblaciones y estudios de estructura de poblaciones.

ESTRUCTURA GENÉTICA

Se entiende por "estructura genética" de una población al tipo, cantidad y distribución de la variación genética presente en la misma, que se expresa en términos de frecuencias génicas o genotípicas. La cantidad y la distribución de la variación genética son las mismas en todas las plantas de los cultivares Híbridos (F_1), cultivares Líneas y cultivares Clones. En cambio, en los cultivares Poblaciones, cultivares Sintéticos, cultivares Multilíneas o Multiclones, la cantidad y distribución de la variación genética es diferente, desde unos pocos individuos distintos (genotipos) hasta llegar a algunos cultivares Poblaciones en alógamas con base genética muy amplia, donde todas las plantas de la población pueden ser genéticamente diferentes, aunque en equilibrio teórico de Hardy-Weinberg. En todas las variantes posibles, sin embargo, las plantas de los cultivares conservan características comunes y en equilibrio, que hacen a los mismos estables y homogéneos, diferentes a los ya existentes, y cumplen con la condición de ser una novedad comercial y tener una denominación. Esto último sintetiza los requerimientos para que una variedad vegetal (cultivar) pueda ser inscripta en el Registro Nacional de Cultivares del INASE (adherido a UPOV ´78) y nótese la importancia que se le da a la estabilidad y a la homogeneidad de los cultivares.

Muchas de las poblaciones de plantas nativas se asemejan a los cultivares Poblaciones en cuanto a estructura genética y variabilidad.

CARACTERES ECOFISIOLÓGICOS

Ante la variación y complejidad descripta más arriba, el aporte de la ecofisiología es necesario y central. En especial, para describir, clasificar y explicar cómo se comportan las plantas de un cultivo en una situación particular. Y esa situación particular puede ser puntual y específica para un genotipo o tener una aplicación más amplia, que dependerá principalmente de la estructura genética de la población en estudio (uno de los cinco tipos de cultivares ya mencionados en el Cuadro 1) y de la especificidad o amplitud del carácter ecofisiológico en cuestión que se desarrollará más adelante. A su vez, puede haber genes con efectos antagónicos fuertemente ligados o genes con efectos pleiotrópicos que pueden afectar dos o más caracteres a la vez. Esos genes pleiotrópicos son los determinantes de efectos fisiológicos complejos sobre el rendimiento, calidad, tolerancia a estreses bióticos y abióticos y muchos caracteres más de importancia agronómica. El rendimiento, tal vez el factor más importante en todos los cultivos, está determinado por numerosos genes (herencia poligénica) que están distribuidos en diferentes cromosomas y con efectos genéticos de lo más variables.

El desarrollo ontogénico de un vegetal se corresponde con un conjunto de fenómenos morfológicos y fisiológicos que se dan en un orden preciso acorde a la información genética de cada individuo y la agronomía como ciencia describe, clasifica y explica mediante la ecofisiología para sentar las bases del manejo agronómico (Tourte 2002). Y el mejoramiento genético junto a la ecofisiología son las disciplinas para lograr mayor producción de los cultivos con sustentabilidad

La interrelación entre Ecofisiología y Genética se analizará en los párrafos siguientes como una forma práctica para incrementar el rendimiento y la calidad de los cultivos y la adaptación de los mismos a condiciones ambientales específicas.

ECOFISIOLOGÍA, MANEJO DE CULTIVOS Y FITOMEJORAMIENTO

Los principales cultivos pueden ser agrupados por sus similitudes o diferencias en base al tipo de cultivar utilizado (ver Cuadro 1) y a las particularidades de la "semilla" (en sentido amplio de órgano reproductivo) utilizada para la multiplicación con un manejo agronómico estándar. En todas esas especies y sus respectivos cultivares hay una estructura genética particular, que el mejorador consecutivamente tiene presente desde que selecciona a los fenotipos en la colección base de selección hasta que obtiene e

inscribe un nuevo cultivar. De igual modo, un ecofisiólogo que estudia caracteres fisiológicos o interacciones del cultivo con factores ambientales, debería tener presente esas estructuras genéticas en el momento que elige los materiales de estudio (tipos morfológicos, cultivares o genotipos) y cuando obtiene sus conclusiones y recomendaciones, ya que los mismos determinarán los alcances de su estudio. Hasta ahora se mencionaron los cultivos, pero lo mismo ocurre, por ejemplo con las estructuras genéticas de las poblaciones de árboles nativos o en silvicultura. Aquí, nuevamente como se mencionó para los cultivos agrícolas más importantes, el ecofisiólogo deberá considerar las estructuras poblacionales con su variabilidad genética en los materiales estudiados, para obtener sus conclusiones y facilitar recomendaciones a la silvicultura.

Veremos primero algunos ejemplos en cultivos extensivos de importancia agronómica y más adelante las diferencias con otros, como los silvopastoriles o los intensivos. Si en algunos de los cultivos importantes nos remontáramos a la especie, siguiéramos con la variedad botánica y llegáramos a la variedad cultivada (cultivar o variedad), veríamos claramente cómo serían los alcances y las limitaciones de los caracteres agronómicos como los morfofisiológicos si los asociáramos al nivel jerárquico o a la unidad experimental en la que se haya realizado el estudio. Así, por ejemplo, Andrade (2005) refiriéndose a las especies en las que desarrollaron sus investigaciones en Ecofisiología, concluye: "Características morfofisiológicas de cada especie determinan la ubicación precisa de estas etapas críticas en el ciclo ontogénico" refiriéndose a las etapas críticas del ciclo ontogénico en las principales especies cultivadas de la región. Basado en los resultados, continúa, encontraron una estrecha correlación positiva entre el rendimiento en grano y el estado fisiológico de los cultivos durante determinados períodos. Este ejemplo es representativo de un alcance amplio de los resultados y en buena medida abarca a casi todos los cultivares de cada cultivo en estudio, al menos como lo describe el autor y como se desprende de la bibliografía de los trabajos de su grupo. En síntesis, esas características morfofisiológicas a las que se refirió Andrade (2005), son más dependientes de especie que dependientes de población (población en el sentido de estructura genética o conjunto de individuos) y por ello tienen un alcance tan amplio que involucra a la especie. En el otro extremo están los estudios que involucran a un solo genotipo, comúnmente a híbridos, líneas o clones de las más variadas especies, que el obtentor quiere evaluar ecofisiológicamente y donde la trascendencia del estudio se remite a un paquete tecnológico agronómico esencial para conocer y difundir el cultivar.

Otro tipo de ensayos de la ecofisiología considera especies que difieren en la plasticidad vegetativa y en la respuesta del rendimiento reproductivo cuando la cantidad de recursos disponibles por individuo varía. Es conocido que hay especies más plásticas que otras (el mismo genotipo adoptando más de un fenotipo), especialmente para algunos caracteres morfofisiológicos particulares. También se conoce que dentro de cada especie hay distintos grados de plasticidad para los mismos caracteres (cultivares dentro de especies), aunque en mucho menor grado que entre especies. Ese menor grado de variabilidad, más compleja para detectarla y fijarla, es justamente unos de los mayores desafíos para los mejoradores. Este ejemplo de plasticidad, entonces, agrupa un fenómeno para dos entidades jerárquicas (1.-entre especies y 2.-cultivares dentro de especies) y permite nuevamente advertir sobre las interpretaciones y alcances entre especies y dentro de especies, cuando de caracteres morfofisiológicos se trate.

Es bastante frecuente en la evaluación agronómica que dentro de una misma especie se evalúen germoplasmas de distinto ciclo ya que tienen consecuencias productivas y de adaptación a diferentes ambientes en especial asociados a períodos críticos. El comentario que haré desde la Genética o el mejoramiento, en la misma línea de lo que estamos analizando, es remarcar que el ciclo es una característica de peso que diferencia al fenotipo, pero que generalmente no todos los materiales del mismo ciclo se comportan de igual manera. Eso determina que el desafío para el mejorador sea encontrar esas diferencias en el germoplasma disponible de un mismo ciclo, los denominados individuos transgresivos. Para ello será necesario detectar comportamientos diferentes dentro de cada ciclo para poder obtener así individuos transgresivos para esos caracteres, que podrán ser la base de nuevos cultivares.

En el siguiente ejemplo en girasol, de alcance más específico, trabajando con 18 líneas endocriadas y refiriéndose a la variabilidad genética, Pereyra Irujo et al (2008) estudiaron la identificación y cuantificación de esa variabilidad para la respuesta del crecimiento foliar al déficit hídrico para proveer de fuentes de tolerancia a estrés hídrico compatibles con distintos sistemas agrícolas. El objetivo de ese trabajo en un grupo de 18 genotipos de girasol, fue analizar la respuesta del crecimiento foliar al déficit hídrico con el fin de identificar y describir

cuantitativamente las fuentes de variabilidad genética para ese carácter. Con respecto al título del trabajo que incluyó el término variabilidad genética en girasol, es necesario hacer algunas aclaraciones ampliatorias antes de analizar la estructura genética involucrada. La variabilidad genética no es más que la medición de la variabilidad en el seno de una población de genotipos (sólo las líneas endocriadas de este estudio), con algunos contrastantes para el carácter, para hacer más evidentes las diferencias y sus causas. Es decir hacer intervenir genotipos para medir la supuesta variabilidad y asociar, con metodología adecuada, la mayor parte de las divergencias a las diferencias entre genotipos. Ese trabajo dividido en cuatro partes comenzó evaluando las 18 líneas hasta evaluar detalladamente al final sólo las dos más contrastantes en la respuesta a la tasa de crecimiento foliar y duración. En ese aspecto, el trabajo pretendió obtener diferencias en el comportamiento a esos caracteres ecofisiológicos entre genotipos o entre grupos de genotipos de girasol, y sólo en 18 o finalmente dos genotipos de girasol. Conviene remarcar que según el germoplasma de origen (también denominado fuente de tolerancia en ecofisiología), los genotipos tuvieron comportamientos más o menos estables, más o menos constantes y más o menos variables según las condiciones del experimento. En esas condiciones y sin entrar en detalle, están asociadas acciones génicas particulares en los genotipos y el valor "per se" de los mismos. Este análisis de ese trabajo, pretende hacer notar la complejidad y particularidad de los fenómenos en los que están involucrados genotipos y advertir, que quien utiliza y aplica estos resultados de la medición de caracteres ecofisiológicos no puede inferir o extrapolar más allá de lo que representan los genotipos del estudio con sus interacciones y condiciones ambientales particulares. Si esos genotipos del estudio fueran líneas paternas de híbridos, no aportarán más que un valor "per se" para los caracteres estudiados y un valor de la aptitud combinatoria general (ACG). Pero ya en el híbrido, la Aptitud Combinatoria Específica (ACE) pasa a ser el parámetro determinante y su estimación revelará efectos génicos epistáticos, o sea de interacción entre genes, que podrían ser diferentes a los medidos en los genotipos paternos (valores "per se") para muchos caracteres agronómicos importantes. Y en consecuencia, habrá que evaluar nuevamente al híbrido para esos caracteres si se quiere saber con precisión el comportamiento del mismo en su interacción con ambientes diversos. Los autores concluyeron acorde a esto último, expresando que las líneas deberían ser combinadas para aumentar la variabilidad en la respuesta positiva al déficit hídrico en girasol. Estamos ante un claro ejemplo de que el estudio de líneas homocigotas en alógamas significa un avance para clasificar y conocer genotipos paternos, pero que el híbrido comercial es una entidad jerárquica y agronómica diferente y que el comportamiento agronómico basado en características ecofisiológicas deberá estar referido a ese híbrido particular.

Lo analizado hasta aquí se puede aplicar tanto a cultivos extensivos como intensivos. En general en los cultivos extensivos las características agroecológicas del país hacen que se presenten condiciones subóptimas de humedad y temperatura en etapas críticas del cultivo. Es por ello que la interacción genotipo-ambiente ha sido muy estudiada tanto para el manejo agronómico y los modelos de simulación como para la selección y el mejoramiento genético. Conviene resaltar aquí que la interacción genotipo-ambiente por sus características y análisis tiene un componente biométrico importante que permite inferir sobre el manejo agronómico y sobre el mejoramiento del cultivo. Para los cultivos intensivos con condiciones más estables de humedad y temperatura, los caracteres ecofisiológicos y sus interacciones tienen más que ver con calidades diferenciales de los productos, precocidad y producciones "primicias", caracteres organolépticos y utilización industrial, aunque la relación entre estructuras genéticas y caracteres ecofisiológicos son las mismas. Para sistemas forestales Kramer (1986) hizo una revisión sobre el rol de la fisiología y afirmó que aunque se haya acumulado una gran cantidad de información sobre la fisiología de los árboles la contribución a la silvicultura fue menor a la esperada. En relación al objetivo del presente trabajo, en un gráfico mostró la relación del potencial genético de los programas de mejoramiento forestal, los factores ambientales bióticos, abióticos y de manejo y los procesos fisiológicos desde el nivel celular hasta el nivel del árbol. También planteó que los genetistas proporcionaran genotipos con una combinación más eficiente para los procesos fisiológicos esenciales. Esto reafirma lo ya señalado sobre la interrelación entre Ecofisiología y Genética que resulta generalizada para todo tipo de cultivos y sistemas productivos agronómicos. Y nuestro trabajo se concibió poniendo énfasis en las estructuras genéticas de esas diferentes poblaciones de plantas, ya que no siempre se consideran las estructuras genéticas cuando se estudian caracteres ecofisiológicos

Un párrafo aparte merece el aporte realizado por el

mejoramiento genético para el rendimiento de los cultivos con la ayuda de las bases conceptuales de la ecofisiología. El aporte del mejoramiento se dio con nuevos y mejores cultivares con ganancias genéticas realizadas por selección mendeliana, con herencias poligénicas complejas que con el aporte de la biometría, los criterios de selección y el "ojo" del mejorador se fijaron en mejores genotipos.Y esto seguirá como eje del mejoramiento hacia adelante, aunque cada vez con más aportes de herramientas biotecnológicas, que cambiarán la magnitud y los alcances del mejoramiento pero no su esencia, ni tampoco los conceptos aquí vertidos.

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MAPEO DE QTL PARA UNA MEDIDA MULTIVARIADA DE LA REACCIÓN AL VIRUS DEL MAL DE RÍO CUARTO

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ABSTRACT

The disease severity index (DSI), a multidimensional indicator of resistance, is a weighted average of the severity and incidence of the disease of maize caused by the Mal de Río Cuarto Virus (MRCV). Interval mapping was used in order to detect *quantitative trait loci (*QTL) associated with the DSI of the Mal de Río Cuarto (MRC) disease in a population of recombinant inbred lines (RILs) of maize tested in environments where the disease is endemic. The results suggest the existence of genomic regions (chromosomes 1, 4, 6, 8 and 10) with a significant effect on MRC. About 40% of the significant intervals were consistent with those identified in previous studies. However, other intervals were associated with the simultaneous expression of symptoms that are not detected when the analysis is performed trait by trait. The results suggest that virus-resistant genotypes can be selected using the particular symptoms of the disease as well as using MRC-QTL.

Key words: maize, recombinant inbred lines, disease severity index, microsatellites, viral disease.

RESUMEN

El índice de severidad de enfermedad (ISE), un indicador de resistencia multidimensional, es una media ponderada de la severidad e incidencia de la enfermedad del maíz causada por el Mal de Río Cuarto Virus (MRCV). En el presente estudio se realizó un mapeo por intervalo con el objetivo de identificar *loci* de caracteres cuantitativos o QTL ("*quantitative trait loci*") asociados con el ISE del Mal de Río Cuarto (MRC) en una población de líneas endocriadas recombinantes (RILs) de maíz evaluadas en ambientes donde la enfermedad es endémica. Los resultados sugieren que algunas regiones genómicas (cromosomas 1, 4, 6, 8 y 10) tienen un efecto significativo sobre la reacción al MRCV. Alrededor del 40% de los intervalos significativos coinciden con aquellos identificados en estudios previos. Por otro lado, se identificaron intervalos no detectados cuando el análisis se realizó carácter por carácter. Los resultados sugieren que es posible seleccionar genotipos resistentes al virus utilizando tanto los síntomas particulares de la enfermedad como QTL asociados al MRC.

Palabras clave: maíz, líneas endocriadas recombinantes, índice de severidad de enfermedad, microsatélites, enfermedad viral.

INTRODUCCIÓN

El Mal de Río Cuarto (MRC) es la patología más importante del maíz en Argentina (Redinbaugh y Pratt, 2009). En años de gran incidencia, se registraron lotes con pérdidas en los rendimientos superiores al 70% (Rodriguez Pardina *et al.*, 1998). El agente causal del MRC, *Mal de Río Cuarto virus* o MRCV por sus siglas en inglés, pertenece al género *Fijivirus* familia *Reoviridae* (Distéfano *et al.*, 2002). Este virus es naturalmente transmitido por medio del insecto vector *Delphacodes kuscheli* en forma persistente, circulativa y propagativa, por lo que el vector es reservorio natural y la población de macrópteros migrantes constituye el principal inóculo de la enfermedad (Ornaghi *et al.*, 1993).

La ocurrencia y magnitud de las enfermedades virales en la naturaleza dependen de la interacción del vector con el patógeno, el hospedante y el ambiente. El control y la prevención de estas enfermedades provienen de la ruptura de alguna de las interacciones en el tetraedro de la enfermedad (Redinbaugh y Pratt, 2009).

La presencia de enaciones sobre las nervaduras en el envés de las hojas constituye el síntoma característico y distintivo del MRC. Debido a las modificaciones en los niveles hormonales endógenos se presentan otros síntomas como tallos achatados con entrenudos cortos, hojas del tercio superior recortadas o reducidas, panojas atrofiadas de escaso tamaño y espigas múltiples con pocos o sin granos (March *et al.,* 1995). El modo más eficaz para controlar enfermedades virósicas en cultivos extensivos es la incorporación de resistencia genética (Di Renzo *et al.*, 2002; Di Renzo *et al.*, 2004; Redinbaugh y Pratt, 2009).

La biología molecular y la bioinformática ayudan a localizar *loci* de caracteres cuantitativos ("quantitative trait loci" o QTL) y permiten evaluar los efectos de genes individuales y las interacciones entre ellos y con el ambiente. La identificación de QTL para diferentes enfermedades y la consecuente selección indirecta favorecen la efectiva acumulación de genes de resistencia cuantitativa (Asíns, 2002; Lucas, 2010). La Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO) promueve la aplicación de la selección asistida por marcadores (*marker-assisted selection* o MAS), la cual es de uso frecuente en los actuales programas de mejoramiento genético de maíz tanto públicos como privados (Ragoty Lee, 2007).

Numerosos trabajos han identificado QTL para resistencia a virus mediante el uso de microsatélites (SSR) (Wu *et al.*, 2007; Ingvardsen *et al.*, 2010). La eficiencia para manipular QTL depende de su identificación y ubicación precisa así como de la consistencia en diferentes generaciones, fondos genéticos y ambientes (Rafalski, 2002). Trabajos realizados con una población de mapeo $F_{2:3}$, de fondo genético similar al del presente estudio, permitieron estimar valores moderados de heredabilidad del carácter resistencia al MRC (Di Renzo *et al.*, 2002) y realizar análisis de asociación que indicaron dos posibles QTLs en los grupos de ligamiento 1 y 8 (Di Renzo *et al.*, 2004). Los resultados obtenidos con una población $F_{2:3}$ de mapeo producto de un cruzamiento biparental diferente al utilizado por Di Renzo *et al.* (2004) permitieron identificar QTL relacionados a los síntomas de MRC en los cromosomas 1, 4, 8 y 10 (Kreff *et al.*, 2006). En un trabajo previo (Bonamico *et al.*, 2010), empleando un método de análisis multivariado, sugerimos la presencia de un grupo reducido de SSR asociados significativamente con diferentes síntomas del MRC. El mapeo de QTL para caracteres asociados a cada uno de los síntomas del MRC (Bonamico, 2010) y para los caracteres incidencia y severidad (Bonamico *et al.*, 2012) permite inferir que el mapeo de un carácter sintético o matemáticamente construido a partir de la combinación de estas dimensiones de análisis constituye una alternativa para seleccionar genotipos resistentes al MRC. Dada la existencia de diversos síntomas que se manifiestan en grados diversos y con distinta incidencia en las poblaciones bajo selección, se ha propuesto el cálculo de índices de resistencia como el índice de severidad de enfermedad (ISE) (Grau *et al.,* 1982). Este índice constituye una media de la severidad de la reacción al MRCV clasificada según la expresión simultánea de los síntomas característicos, ponderada por la incidencia de los distintos grados de severidad en un conjunto de plantas del genotipo (Di Renzo *et al.*, 2004). La identificación de QTL asociados con el ISE y su validación con QTL previamente mapeados para resistencia al MRC en maíz es crucial para poder desarrollar estrategias efectivas de mejoramiento genético. Estudios de validación han revelado que hay pocos QTL comunes entre diferentes generaciones de poblaciones de mapeo y entre ambientes de evaluación en una misma generación (Melchinger *et al.*, 1998).

Los objetivos de este trabajo fueron: i) mapear QTL asociados al ISE para la reacción al MRCV en una población de líneas endocriadas recombinantes de maíz; ii) analizar la consistencia entre las regiones genómicas aso-

ciadas al ISE y las identificadas con otras formas de estimar la reacción al MRCV.

MATERIALES Y MÉTODOS

Material vegetal

Una población de líneas endocriadas recombinantes (RILs) ($F_{2:6}$) de maíz derivadas del cruzamiento entre la línea BLS14 (resistente) y la línea Mo17 (susceptible) se evaluó por su reacción a la infección del MRCV mediante caracteres fenotípicos relacionados con la expresión de la enfermedad. Estas RILs son descendientes de la población $F_{2:3}$ utilizada en Di Renzo *et al*. (2004) para un estudio de mapeo de QTL para resistencia al MRC.

Ensayo de campo

Los ensayos de campo se realizaron en un diseño en bloques completos al azar (DBCA) con dos repeticiones, bajo condiciones de transmisión natural en dos localidades de la zona sur de la provincia de Córdoba: Río Cuarto (33° 8'S, 64° 20'O, 334 msnm, 630 mm de precipitación anual) y Sampacho (33° 19'S, 64° 42'O; 510 msnm, 676 mm de precipitación anual). Los ensayos se establecieron en los ciclos agrícolas 2004/05, 2005/06 y 2006/07. Cada una de las seis combinaciones localidad-año se consideró un ambiente: Río Cuarto 2005 (R5), Río Cuarto 2006 (R6), Río Cuarto 2007 (R7), Sampacho 2005 (S5), Sampacho 2006 (S6) y Sampacho 2007 (S7). Las líneas parentales se incluyeron en cada ambiente. El número de plantas osciló entre 10 y 15 espaciadas a 20 cm sobre el surco. Las labores de presiembra fueron las convencionales. Las malezas preemergentes y postemergentes fueron controladas con agroquímicos y en forma manual, respectivamente.

Evaluación fenotípica

La reacción a la infección del MRCV de cada una de las RILs se estimó mediante el ISE. Para calcular este índice, las plantas de cada genotipo fueron clasificadas según el grado de severidad de enfermedad propuesto por Ornaghi *et al*. (1999), expresado en una escala que consta de cuatro grados (0, 1, 2 y 3) según la simultaneidad de síntomas observados. El grado 0 corresponde a una planta asintomática; el grado 1 a una planta que presenta enaciones, con o sin disminución de altura; el grado 2 a una planta con presencia de enaciones, reducción del tamaño de la panoja, láminas foliares atrofiadas en el tercio superior, y espigas generalmente curvas y pequeñas; y el grado 3 a una planta con síntomas muy severos, enaciones, acortamiento de entrenudos, producción de granos por espiga escasa o nula, y espigas pequeñas, múltiples y sin granos. Grau *et al*. (1982) propusieron la siguiente ecuación para el cálculo del ISE, que puede tomar valores de 0 a 100, siendo este último valor el correspondiente a genotipos severamente afectados:

$$\text{ISE} = \sum_{i=0}^{3} \left(\frac{\text{gradoi} \times \text{nº de plantas en el gradoi}}{\text{nº total de plantas} \times 3} \right) \times 100$$

Evaluación genotípica

El ADN de las RILs se extrajo a partir de tejido foliar liofilizado de plantas de maíz jóvenes crecidas en invernáculo, según el método utilizado en Di Renzo *et al*. (2004). Un conjunto de 150 marcadores moleculares SSR se evaluó en las líneas parentales BLS14 y Mo17. Del total de marcadores, 113 revelaron bandas nítidas y de éstos sólo los polimórficos se utilizaron para analizar la población de RILs. Para la construcción del mapa de ligamiento, se eligieron los marcadores polimórficos con el menor número de datos faltantes. Los perfiles de ADN obtenidos para cada RIL correspondieron a marcadores SSR pertenecientes a ocho cromosomas de maíz (1, 2, 3, 4, 6, 8, 9 y 10). El 50% de los SSR probados pertenecen a los grupos de ligamiento 1, 4, 8 y 10, en los que se detectaron QTL para resistencia al MRC (Di Renzo *et al*., 2004; Kreff *et al*., 2006). Las secuencias de los marcadores SSR se obtuvieron a partir de la base de datos genéticos de maíz (MaizeGDB, http://www.maizegdb.org) y se sintetizaron en el laboratorio Alpha DNA (http://www.alphadna.com). La amplificación por PCR se realizó según el protocolo utilizado en Di Renzo *et al*. (2004).

Mapeo de QTL

El mapeo de los QTL se realizó con el programa Plabqtl versión 1.0 (Utz y Melchinger, 1995) usando un mapa genético construido a partir de la misma población de RILs (Bonamico *et al*., 2012). Los análisis se efectuaron por ambiente y a partir del ensayo multiambiental, usando los datos fenotípicos de cada ambiente, se ajustó primero un modelo lineal mixto (MLM) (Littell *et al*., 2006) para obtener los estimadores del ISE de cada línea, corregidos por posibles correlaciones experimentales. Este modelo incluyó el efecto de bloque como fijo y una matriz de varianza y covarianza residual con estructura espacial de tipo exponencial para contemplar la posible auto-correlación entre

parcelas dentro de cada ambiente. No obstante, la comparación de este modelo con el modelo de independencia sugirió que las medias aritméticas de cada genotipo en cada ambiente podían ser usadas en los análisis subsecuentes. Con estas medias se emplearon los métodos de mapeo por intervalo simple (SIM, Modelo III) y por intervalo compuesto (CIM, Modelo II) (Zeng, 1994). En el CIM se utilizaron marcadores moleculares como cofactores. El logaritmo de las probabilidades (*log of the odds* o LOD) mínimo para declarar la presencia de un posible QTL fue 2.5. La posición del QTL fue asignada al punto del intervalo de análisis donde el valor de LOD fue máximo. El efecto genético de aditividad fue estimado como la mitad de la diferencia entre el valor de los dos homocigotas, asumiendo que el segundo parental es quien aporta alelos favorables para la variable en estudio (Falconer, 1989). Valores negativos indican que el parental BLS14 aporta alelos favorables para disminuir el ISE. La variación fenotípica total del ISE, explicada por los posibles QTL detectados, se estimó a través de un modelo de regresión múltiple.

RESULTADOS

Evaluación fenotípica

El ISE de las RILs mostró una distribución de frecuencias próxima a la normal al considerar el conjunto de ambientes de evaluación (Figura 1). El valor medio en las RILs fue aproximadamente intermedio al de los parentales. El valor medio del ISE en el parental resistente fue menor al 10%, mientras que el parental susceptible presentó un valor superior al 80% del máximo de la escala.

La Tabla 1 muestra los valores del ISE del MRC obtenidos en las líneas BLS14 (resistente) y Mo17 (susceptible) y las RILs derivadas ($F_{2:6}$), en seis ambientes de evaluación. El ensayo sembrado en la localidad de Río Cuarto en la campaña 2006/07 (R7) bajo condiciones de transmisión natural presentó infecciones severas, mientras que los ensayos realizados en la misma localidad en las campañas 2004/05 (R5) y 2005/06 (R6) escaparon al pico poblacional de los vectores de la enfermedad. Como resultado, en estos últimos dos ambientes, los materiales susceptibles no manifestaron síntomas tan severos. Los materiales evaluados en Sampacho durante los ciclos agrícolas 2004/05 (S5), 2005/06 (S6) y 2006/07 (S7) presentaron un adecuado nivel de síntomas. En el ciclo agrícola 2006/07 se observaron lotes en la zona en donde la enfermedad es endémica con un 100% de plantas afectadas por el MRCV. La Secretaría de Agricultura de la provincia de Córdoba estimó pérdidas económicas por MRC durante dicha campaña de 70 millones de dólares (Mattio *et al.*, 2008).

En general se observaron correlaciones positivas y significativas entre el ISE y distintos síntomas del MRC, lo cual indica que a mayor valor de afección en cada uno de los síntomas, mayores los valores del ISE (Tabla 2). Los valores de correlación más elevados con el ISE, en los diferentes ambientes, fueron estimados para presencia de enaciones y acortamiento de entrenudos. Estos síntomas serían los recomendables para la selección ambiente-específica de genotipos de maíz resistentes al MRC cuando no se calcule el ISE.

Mapeo de QTL

Siete QTL fueron detectados para el ISE. En Sampacho 2005 se identificaron tres QTL y en Sampacho 2007 cuatro. En los ambientes Río Cuarto 2005, Río Cuarto 2007 y Sampacho 2006 se identificaron dos QTL significativos, mientras que en Río Cuarto 2006 se identificó sólo un QTL (Tabla 3).

Los siete QTL identificados para el ISE pertenecen a los cromosomas 1, 4, 6, 8 y 10. En los cromosomas 1 y 4 se identificaron dos QTL, mientras que en los cromosomas 6, 8 y 10 se identificó sólo un QTL en cada uno. De los siete QTL detectados, que se localizaron en los cromosomas 1 (bin 1.03 y 1.07), 4 (bin 4.03 y 4.05), 6 (bin 6.02), 8 (bin 8.03) y 10 (bin 10.02), dos fueron identificados de manera específica en los ambientes R6 y S5 en los cromosomas 4 (bin 4.05) y 10 (10.02), respectivamente.

En los ambientes R7, S5 y S7 prevaleció un mayor valor de afección de enfermedad reflejado en un mayor valor medio del ISE en las RILs. En relación a los marcadores SSR ligados a QTL para resistencia al MRC se observaron regiones del genoma asociadas con la resistencia al MRC de manera exclusiva con los ambientes de mayor y menor valor del ISE, respectivamente. En el grupo de ambientes de mayor ISE, se identificó en forma exclusiva un QTL en el cromosoma 4 (bin 4.03). En el grupo de ambientes de menor ISE, se identificó de manera exclusiva un QTL en el cromosoma 4 (bin 4.05). Por lo mencionado, se infiere que existen factores genéticos diferenciales en la regulación del carácter resistencia al MRC frente a diferentes niveles de ISE.

El porcentaje de variación fenotípica explicada en forma individual por cada QTL, tanto en ambientes indivi-

duales como a través de ambientes, fue de entre 5 y 13%. La variación fenotípica total explicada en forma conjunta por todos los posibles QTL detectados en cada uno de los ambientes individuales varió entre 5 y 18%. Uno de los QTL con mayor efecto sobre el ISE se identificó en el ambiente R7 en el cromosoma 1 (bin 1.03). Este QTL fue significativo de manera consistente en la mayoría de los ambientes de evaluación. Otros dos QTL en el ambiente S7, ubicados en los bins 4.03 y 6.02, explicaron en forma individual un máximo del 13% de la variación fenotípica del ISE.

Dado que se utilizaron RILs, sólo se estimaron los efectos genéticos de aditividad y de epistasis correspondiente, no siendo estos últimos estadísticamente significativos. La estimación de los efectos genéticos no evaluados podría ser de importancia menor ya que el híbrido entre las dos líneas parentales se comportó con una resistencia intermedia entre las mismas (datos no mostrados), lo que sugiere acción génica de tipo aditiva para el carácter.

Los QTL identificados como significativamente asociados con el ISE presentaron inconsistencia en los ambientes evaluados. No obstante, las regiones de los cromosomas 1, 4 y 8 (bins 1.03, 4.03 y 8.03) fueron consistentes en el 50% de los ambientes, incluso el del bin 1.03 en el análisis a través de ambientes. Los QTL identificados en el análisis a través de ambientes, pertenecientes a los cromosomas 1 y 6 (bins 1.03 y 6.02), presentaron interacción QTL×ambiente estadísticamente significativa ($p \leq 0.01$), es decir que no fueron detectados en todos los ambientes evaluados.

Los resultados del presente trabajo permitieron identificar regiones con efecto significativo sobre la reacción a la enfermedad MRC similares o cercanas a las informadas en trabajos anteriores (Di Renzo *et al.* 2004; Bonamico, 2010; Bonamico *et al.*, 2010, 2012) (Tabla 4).

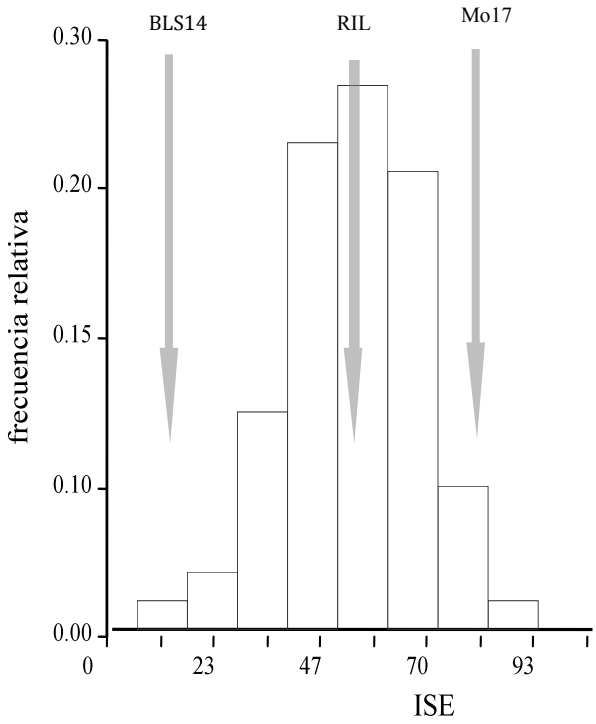

Figura 1. Frecuencia de distribución relativa del índice de severidad de enfermedad (ISE) del Mal de Río Cuarto en una población de líneas endocriadas recombinantes derivadas del cruzamiento entre las líneas BLS14 (resistente) × Mo17 (susceptible), evaluada en seis ambientes en donde la enfermedad es endémica.

	Ambiente					
Genotipo	R5	R6	R7	S5	S6	S7
Mo17	57 ± 4	65 ± 6	99 ± 1	83 ± 4	94 ± 6	95 ± 1
BLS14	3 ± 1	6 ± 1	14 ± 4	11 ± 3	10 ± 1	13 ± 1
RIL	40 ± 1	39 ± 1	87 ± 1	79 ± 1	53 ± 2	78 ± 5

Tabla 1. Valores del índice de severidad de enfermedad (ISE) del Mal de Río Cuarto obtenidos en las líneas BLS14 (resistente) y Mo17 (susceptible) y en las líneas endocriadas recombinantes derivadas (F2:6). Observaciones en seis ambientes en donde la enfermedad es endémica.
R5: Río Cuarto 2005; R6: Río Cuarto 2006; R7: Río Cuarto 2007; S5: Sampacho 2005; S6: Sampacho 2006 y S7: Sampacho 2007.

	Síntomas						
		hoja					
Ambiente	Panoja	largo	ancho	borde	Enaciones	Entrenudos	Espiga
R5	0.36 ***	0.44 ***	0.33 ***	0.54 ***	0.97 **	0.77 **	0.82 **
R6	0.69 **	0.59 ***	0.59 ***	0.72 ***	0.98 **	0.92 **	0.82 **
R7	0.75 **	0.78 **	0.78 **	0.69 **	0.83 **	0.88 **	0.70 **
S5	0.66 **	0.23 ***	0.25 ***	0.24 ***	0.91 **	0.79 **	0.54 **
S6	0.65 **	0.55 **	0.28 ***	0.52 ***	0.96 **	0.91 **	0.62 **
S7	0.58 **	0.35 ***	0.18 *	0.54 ***	0.93 **	0.92 **	0.63 **

Tabla 2. Coeficientes de correlación entre el índice de severidad de enfermedad (ISE) y caracteres asociados a síntomas del Mal de Río Cuarto en seis ambientes en donde la enfermedad es endémica.
R5: Río Cuarto 2005; R6: Río Cuarto 2006; R7: Río Cuarto 2007; S5: Sampacho 2005; S6: Sampacho 2006 y S7: Sampacho 2007. **p<0.01; ***p<0.001.

Cromosoma. bin	Ambiente	Intervalo	Posición	Aditividad		Parental	LOD		R^2	QTL× ambiente
1.03	R5	umc1021-bnlg1866	15	-0.368	ns	BLS14	2.57	b	7.8	
	R7	bnlg1866-phi095	5	-0.161	**	BLS14	2.59	ab	13.2	
	S5	umc1021- bnlg1866	4	0.132	*	Mo17	2.52	b	8.0	
	A través	umc1021-bnlg1866	5	-0.186	*	BLS14	2.72	ab	11.7	**
1.07	S6	bnlg1083-bnlg1556	2	-0.322	**	BLS14	3.06	ab	11.0	
	S7	bnlg1083-bnlg1556	5	-0.240	ns	BLS14	2.56	b	5.6	
4.03	R7	nc004-phi021	1	-0.116	*	BLS14	2.59	b	8.9	
	S5	nc004-phi021	1	-0.206	*	BLS14	2.65	b	9.2	
	S7	nc004-phi021	8	-0.672	ns	BLS14	2.64	b	13.3	
4.05	R6	umc1088-nc005	1	0.292	*	Mo17	2.85	b	9.6	
6.02	S7	bnlg1426-bnlg1371	20	-0.673	ns	BLS14	2.57	b	13.1	
	A través	bnlg1426-bnlg1371	9	-0.222	ns	BLS14	2.56	ab	5.4	**
8.03	R5	umc1741-b1352	20	-0.315	ns	BLS14	2.51	b	4.7	
	S6	umc1741-b1352	0	-0.240	*	BLS14	2.61	ab	6.2	
	S7	phi115-umc1741	3	-0.464	ns	BLS14	2.80	b	9.6	
10.02	S5	umc1556-phi063	0	-0.174	ns	BLS14	2.65	b	9.5	

Tabla 3. Localización y efectos de QTL para el índice de severidad de enfermedad (ISE) del Mal de Río Cuarto en ambientes individuales y a través de ambientes, en donde la enfermedad es endémica.
Ambiente: R5: Río Cuarto 2005; R6: Río Cuarto 2006; R7: Río Cuarto 2007; S5: Sampacho 2005; S6: Sampacho 2006 y S7: Sampacho 2007. Intervalo: referido al mapa publicado en Bonamico et al. (2012). Posición: distancia del QTL (cM) al marcador más próximo. Aditividad: efecto estimado como la mitad de la diferencia entre el valor de los dos homocigotas. Un valor negativo indica que el parental BLS14 aporta alelos para disminuir el valor del ISE. Nivel de significación, ns: no significativo; *<0.05; **<0.01. Letras a y b que acompañan al valor de LOD indican mapeo por intervalo simple (SIM) y compuesto (CIM), respectivamente.
R^2: proporción (%) de la variación fenotípica explicada.

Parental		Población	Carácter	*Loci*	Cromosoma (bin)	Método	Referencia
S	R	de mapeo					
Mo17	BLS14	$F_{2:3}$	ISE	Dos QTLs	1 (1.03), 8 (8.03/4)	CIM	Di Renzo *et al.* (2004)
				Siete SSR	1 (1.03, 1.04), 8 (8.02, 8.06)	ANAVA	
_	_	$F_{2:3}$	Síntomas	Cinco QTLs	1 (1.02/03), 1 (1.07/08), 4 (4.05/7), 8 (8.02), 10 (10.04/5)	CIM	Kreff *et al.* (2006)
Mo17	BLS14	RIL	Síntomas	Doce QTLs	1 (1.01, 1.03, 1.04 1.06), 4 (4.03, 4.05, 4.08), 6 (6.00/2, 6.05), 8 (8.03, 8.08), 10 (10.02)	SIM/CIM	Bonamico (2010)
Mo17	BLS14	RIL	Síntomas	Ocho SSR	1 (1.02, 1.06), 2 (2.06), 6 (6.02), 8 (8.08)	AD	Bonamico *et al.* (2010)
Mo17	BLS14	RIL	INC y SEV	Cuatro QTLs	1 (1.01, 1.06), 4 (4.08), 10 (10.02)	SIM/CIM	Bonamico *et al.* (2012)
Mo17	BLS14	RIL	ISE	Siete QTLs	1 (1.03, 1.06), 4 (4.03, 4.05), 6 (6.02), 8 (8.03), 10 (10.02)	SIM/CIM	En este estudio

Tabla 4. Identificación de QTL y marcadores moleculares SSR asociados con la reacción al virus Mal de Río Cuarto.
Parental: S = Susceptible y R = Resistente.
Carácter: ISE = índice de severidad de enfermedad; INC = incidencia de enfermedad; SEV = severidad de enfermedad.
Método: SIM = Mapeo por intervalo simple; CIM = Mapeo por intervalo compuesto; ANAVA = Análisis de la varianza; AD = Análisis discriminante discreto.

DISCUSIÓN

Evaluación fenotípica

La ocurrencia no sistemática de valores extremos de incidencia y severidad del MRC es común a través de los años. Severas epidemias de esta enfermedad ocurren cuando coinciden la presencia de genotipos susceptibles en estadios sensibles, un abundante reservorio del virus y altas densidades del insecto vector (Harpaz, 1972). La expresión de síntomas severos se relaciona con el estado fenológico del maíz al momento en que se produce la transmisión del virus y la máxima expresión de la enfermedad se da cuando genotipos susceptibles son infectados en estado de coleoptile a 3-4 hojas (March *et al.*, 1995). Acorde a los rangos de valores, la segregación para el carácter fue de tipo transgresiva, es decir que algunas líneas se comportaron más susceptibles que Mo17 y otras más resistentes que BLS14. En trabajos realizados con generaciones tempranas $F_{2:3}$ provenientes del mismo cruzamiento biparental se observó igual tipo de segregación (Di Renzo *et al.*, 2002).

Mapeo de QTL

En maíz fueron identificados numerosos genes y/o QTL para resistencia a patógenos como hongos, bacterias y virus. Los cromosomas 1 y 4 de maíz contienen el mayor número de genes y/o QTL para resistencia a enfermedades causadas por estos patógenos. En los demás cromosomas también se identificaron genes y/o QTL para resistencia aunque en menor cantidad (Wang *et al.*, 2007).

En estudios de resistencia a enfermedades, es frecuente la identificación de QTL ambiente-específicos, es decir que no son detectados en todos los ambientes de evaluación, lo cual puede ser valioso para la mejora genética si se consideran características ambientales particulares (Paterson *et al.*, 1991).

Estudios previos de herencia y mapeo de QTL para resistencia al MRC han indicado que la expresión del carácter está determinada por efectos genéticos tanto aditivos como no aditivos (Presello *et al.*, 1995; Di Renzo *et al.*, 2004). El efecto genético aditivo de cada QTL indicó que los alelos favorables fueron aportados por ambas líneas parentales, tal como se reportó en Di Renzo *et al.* (2004). El parental tolerante al MRC aportó alelos que disminuyeron el ISE en seis de los siete posibles QTL identificados. Estos QTL correspondieron a los cromosomas 1, 4, 6, 8 y 10 (bins 1.03, 1.07, 4.03, 6.02, 8.03 y 10.02). En el bin 1.03, el parental resistente aportó alelos favorables cuando el marcador SSR bnlg1866 se mostró ligado de manera estadísticamente significativa al QTL.

Al comparar los QTL identificados para el ISE con los identificados en estudios previos para cada uno de los otros caracteres relacionados a síntomas del MRC, pueden observarse coincidencias tanto en posiciones como en la proporción de la variación fenotípica explicada. Se ha sugerido que caracteres altamente correlacionados tienen más posibilidades de compartir QTL (Lisec *et al.*, 2008). En un trabajo previo (Bonamico, 2010), reportamos dos QTL para la presencia de enaciones y acortamiento de entrenudos, ubicados en los cromosomas 1 (bin 1.03) y 6 (bin 6.02), de los siete detectados para el ISE. Es decir que alrededor del 40% de los *loci* significativamente asociados con la reacción al MRCV fueron compartidos cuando se identificaron QTL para síntomas individuales o para el índice multidimensional. Estos QTL, detectados en la mitad de los ambientes evaluados, permitieron estimar el ISE y explicaron un máximo del 13% de la variación fenotípica observada. Por otra parte, se detectaron intervalos significativamente asociados con el ISE que no están relacionados con ninguno de los síntomas por sí solos (bins 1.07, 4.03, 4.05, 8.03 y 10.02). En el conjunto de los ambientes, los QTL comunes para el ISE y para síntomas individuales (Bonamico, 2010) se presentaron con correlaciones fenotípicas menores. Esto podría deberse a la existencia de un control genético diferencial o a una alta influencia de factores ambientales. Dado que se identificaron al menos dos QTL comunes entre el ISE, la presencia de enaciones y el acortamiento de entrenudos, una estrategia simple que podría resultar de interés a los mejoradores para seleccionar resistencia al MRC es usar estas variables fenotípicas de fácil y rápida evaluación. No obstante, el ISE es la variable más recomendable para mapear QTL para reacción a la infección del MRCV, debido a que en éste se considera tanto la severidad como la incidencia de la enfermedad.

Un aspecto importante en el uso de QTL para realizar selección indirecta es la consistencia de las posiciones de los QTL observadas en diferentes poblaciones de mapeo (Rafalski, 2002). En un trabajo previo (Di Renzo *et al.*, 2004) donde se evaluó, mediante ISE, una generación temprana con el mismo fondo genético que la población utilizada en este trabajo, se identificaron dos QTL con efectos significativos sobre resistencia al MRC en los cromosomas 1 y 8 (bins 1.03 y 8.03/4). Posteriormente, al mapear el grado medio de severidad y otros caracteres

relacionados a síntomas del MRC, se identificaron doce posibles QTL en los cromosomas 1 (bins 1.01, 1.03, 1.04, 1.06), 4 (bins 4.03, 4.05, 4.08), 6 (bins 6.00/2, 6.05), 8 (bins 8.03, 8.08) y 10 (bin 10.02) mediante el análisis de mapeo por intervalo, utilizando una población de RILs de maíz (Bonamico, 2010). También se identificó un grupo reducido de marcadores SSR significativos en los cromosomas 1 (bins 1.02, 1.06), 2 (bin 2.06), 6 (bin 6.02) y 8 (bin 8.08) mediante un análisis multivariado (Bonamico *et al.*, 2010). El mapeo de QTL para los caracteres incidencia y severidad permitió identificar cuatro QTL localizados en los cromosomas 1, 4 y 10 (Bonamico *et al.*, 2012). Al comparar los resultados con los informados por Kreff *et al.* (2006), quienes trabajaron con una población de mapeo de fondo genético distinto al utilizado en el presente estudio, se observa consistencia en varios de los QTL detectados (Tabla 4). Estos autores informaron que los QTL para resistencia al MRC están ubicados en los cromosomas 1, 4, 8 y 10. No obstante, se encontraron variaciones respecto a los resultados obtenidos por Kreff *et al.* (2006), ya que para el carácter ISE se identificaron dos nuevos QTL no informados por estos autores, en los bins 6.02 y 10.02. La similitud de los resultados obtenidos con los de trabajos previos no es una característica común ya que la acción de diversos factores como el uso de diferentes parentales, el tipo y el tamaño de la población de mapeo, la metodología empleada en la detección de QTL, y las diferentes condiciones climáticas y de cultivo usualmente atentan contra la consistencia de los resultados (Nair *et al.*, 2005).

McMullen y Simcox (1995) sugirieron que, en el genoma de maíz, los genes de resistencia a diferentes patógenos frecuentemente se ubican agrupados e indican regiones genómicas involucradas en la expresión de resistencia a virus en los cromosomas 1 y 6. Otras revisiones recientes en maíz también sugieren que los genes y *loci* que confieren resistencia a diferentes virus no están distribuidos al azar en el genoma. Wisser *et al.* (2006) indicaron la presencia de dos grupos tanto en el cromosoma 1 como en el cromosoma 3, y un grupo en cada uno de los cromosomas 2, 4, 5 y 10. Redinbaugh y Pratt (2009) mencionaron cuatro grupos en los cromosomas 1, 3, 6 y 10. El presente trabajo sugiere que los QTL asociados con la reacción al MRCV, cuantificada a través del ISE, están en los cromosomas 1, 4, 6, 8 y 10. Estos resultados favorecen al desarrollo de conocimientos que son requeridos para la implementación de la selección indirecta mediada por marcadores (MAS) en el mejoramiento de la resistencia a la virosis MRC en maíz.

Alrededor del 43% de los intervalos detectados como significativos fueron los mismos que aquellos identificados en estudios previos que usaron cada unos de los síntomas como caracteres de mapeo (Bonamico, 2010). No obstante, surgieron algunos otros intervalos que estuvieron asociados con la expresión simultánea de los síntomas y que no se detectaron al realizar el análisis carácter por carácter. Por ello, la opción que resulta recomendable es la selección de genotipos resistentes vía marcadores asociados al ISE del MRC.

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RESPIRATORY FUNCTION IN HEALTHY FIRST-DEGREE RELATIVES OF ASTHMATIC ADOLESCENTS

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ABSTRACT

Asthma is a complex disease associated with biological and physiological phenotypes. The objective of this study was to compare the respiratory function in healthy first-degree relatives of asthmatic and non-asthmatic adolescents. We used a cross-sectional approach to assess 101 family cases (presence of one or more adolescents classified as asthmatic) and 275 stable families (families without adolescents classified as asthmatic). We obtained a final sample of 822 relatives. The respiratory function was evaluated by the forced expiratory volume in 1 second (FEV), and the forced vital capacity (FVC), using the *Microlab 3300® Spirometer*. A self-administered questionnaire was used to record upper and lower airway symptoms, allergic symptoms and medical history. To compare continuous variables, the Mann-Whitney and Kruskal-Wallis tests were applied when variables did not follow a Gaussian distribution and the variances were not homogeneous. A linear regression adjusted for age, gender and height was also applied to compare the lung function between asthmatic and non-asthmatic relatives. Parents of asthmatic adolescents had significantly lower values of the lung function than both parents together and mothers of non-asthmatics (84.6 vs. 97.6, $p<0.01$, for FEV and 84.3 vs. 97.9, $p<0.01$, for FVC, and 97.3 vs. 109.7, $p<0.01$, for FEV and 89.5 vs. 105.5, $p<0.01$, for FVC, respectively). Also, siblings of asthmatic adolescents had lower FEV (98.6 vs. 109.4, $p<0.01$) and FVC values (85.9 vs. 102.7, $p<0.01$). The healthy first-degree relatives of asthmatic adolescents have worse respiratory function than those of non-asthmatic adolescents. Asymptomatic relatives of asthmatics can have physiological characteristics that may reveal the phenotypic pattern of the disease.

Key words: Asthma, Respiratory function, Genetic factors, Environmental factors, Phenotype

INTRODUCTION

The familial aggregation of asthma has been documented in several studies (Bedolla-Barajas *et al.*, 2013; Lima *et al.*, 2012; Webster *et al.*, 2011; Wechsler *et al.*, 2002; Chen *et al.*, 2001; Jenkins *et al.*, 1997). However, most of the attention has been focused on the diagnosis and phenotypic characterization of the disease (Thomsen *et al.*, 2010). Despite the attention received, deciphering the relative importance of genetic factors in the origin of asthma has been proved to be a very complex task. Segregation studies have shown that there are genes that can explain the development and outcome of the disease (Koppelman *et al.*, 2002; Chen *et al.*, 2001; Los *et al.*, 1999). Studies made with twins and analyzing genealogical trees have allowed the observation of the clear input of genetic factors in the disease (Fagnani *et al.* 2008; Rasanen *et al.*, 2000). In a research involving 6,996 pairs of twins, the concordance for asthma was 19% in monozygotic pairs and 4.8% in dizygotic ones (Nolan, 1994).

The wide phenotypic variability found among asthma patients suggests an important etiological heterogeneity, strengthened by environmental influences (Abdou *et al.*, 2013; Ober *et al.*, 2011; Thomsen *et al.*, 2010).

A specific gene located on chromosome 11 seems to be responsible for the transmission of atopy, which occurs through the maternal line (Holberg *et al.*, 1998; Nolan, 1994; Cookson, 1992). However, the development of asthma is more dependent on environmental factors, which act in individuals with genetic susceptibility (Colilla *et al.*, 2003; Howard *et al.*, 2003; Palmer *et al.*, 2000). This is supported by the fact that people from regions with low prevalence of asthma, such as isolated rural environments, when set in urban areas, end up expressing an increased frequency of the disease, possibly due to factors such as exposure to certain new fungi, infectious agents, air pollution and changes in the eating patterns (Gonzalez-Barcala *et al.*, 2012; Ober *et al.*, 2011; Delfino *et al.*, 2003; Custovic *et al.*, 1996; Nicolai *et al.*, 1997). Studies carried out after the reunification of Germany have shown that the prevalence of asthma and other respiratory problems tends to reach values close to those of western cities (Heinrich *et al.*, 1998).

Although the presence of atopy as a familial antecedent is more common among asthmatics, it has been suggested that the genetic determinants of atopy and asthma are different (Kurzius-Spencer, *et al.* 2012).

Children whose family members have a history of asthma are more likely to have the disease, particularly if both parents are asthmatic (Burke *et al.*, 2003; Kreeger, 2003; Fernández-Espinar *et al.*, 2001; Barros *et al.*, 1999; Arshad *et al.*, 1993). Beyond the demonstration of the familial aggregation of asthma, familial aggregation of the disease indicators and of the measured and predicted values of respiratory function parameters for patients and their descendants has also been observed (Bedolla-Barajas *et al.*, 2013; Holberg *et al.*, 1998). This aggregation can also be explained by genetic and environmental factors, as well as by lifestyle habits, including smoking (Lima, *et al.*, 2012; Lewitter *et al.*, 1984). However, the analysis of this aggregation implies greater complexity due to the simultaneous influence of these factors, whose relative contribution is difficult to quantify.

The objective of this study was to compare the respiratory function in healthy fist-degree relatives of asthmatic and non-asthmatic adolescents.

MATERIALS AND METHODS

Using the school population of the district of Viseu (Portugal), a randomly selected sample of 101 families with adolescents with asthma criteria (presence of episodes of dyspnea, with wheezing in the absence of respiratory infection) was selected. For each family with adolescents with asthma, two or more families with adolescents without asthma criteria and living in the same street or locality as the asthmatic families were selected. We assessed a total of 101 family cases (presence of one or more asthmatic adolescents) and 275 stable families (families without asthmatic adolescents), obtaining a final sample of 822 individuals. The criteria for inclusion of adolescents in the analysis were: to be aged between 12 and 18 years old, not having smoked for 6 months straight and absence of history of asthma or bronchitis.

A phone call was made to explain the objectives of the study and to book the day and time of the interviews. The adolescents and first-degree relatives living under the same roof (father, mother and siblings over twelve years of age) were interviewed by home visit. When any of these individuals with criteria were not at home at the time of this visit, we agreed a new date for a second home visit. Their not being home in the second visit determined their exclusion of participation in the study. A self-administered questionnaire was filled out by the adolescents and their

relatives to collect information regarding upper and lower airway symptoms, allergic symptoms and medical history and then the forced expiratory volume in 1 second (FEV) and the forced vital capacity (FVC) were evaluated with the *Microlab 3300® Spirometer*. FEV was calculated using the formula (100x FEV observed / expected FEV) and FVC by the formula (100x FVC observed / predicted FVC). The respiratory function tests were performed in a sitting position, repeated at least three times and no more than five times, considering the most correct and highest value. Before each assessment, data regarding sex, age, height and ethnicity were introduced into the spirometer. The spirometer was calibrated daily.

All the participants were weighed barefoot, without coats and with the same digital scale. Height was measured using a tape measure. Asthma and bronchitis were assessed through the question "Has your doctor ever told that you that you had asthma or bronchitis?". Smoking habits were assessed through questions concerning the status of "smoker", "non-smoker" and "ex-smoker." Every participant who smoked at least one cigarette a day and had not stopped smoking in the last six months was considered a smoker.

Data were processed and analyzed using the Epi-Info 6.04® and the Statistical Program for the Social Studies (SPSS 11.5®). Continuous variables were described by the mean value and the standard deviation. To compare continuous variables, we decided to apply the Mann-Whitney and Kruskal-Wallis tests when the variables did not present a Gaussian distribution and the variances were not homogeneous. A linear regression adjusted for age, gender and height was also applied to compare the lung function between asthmatic and non-asthmatic relatives. To analyze the variation of a quantity depending on another, we used the Pearson correlation coefficient.

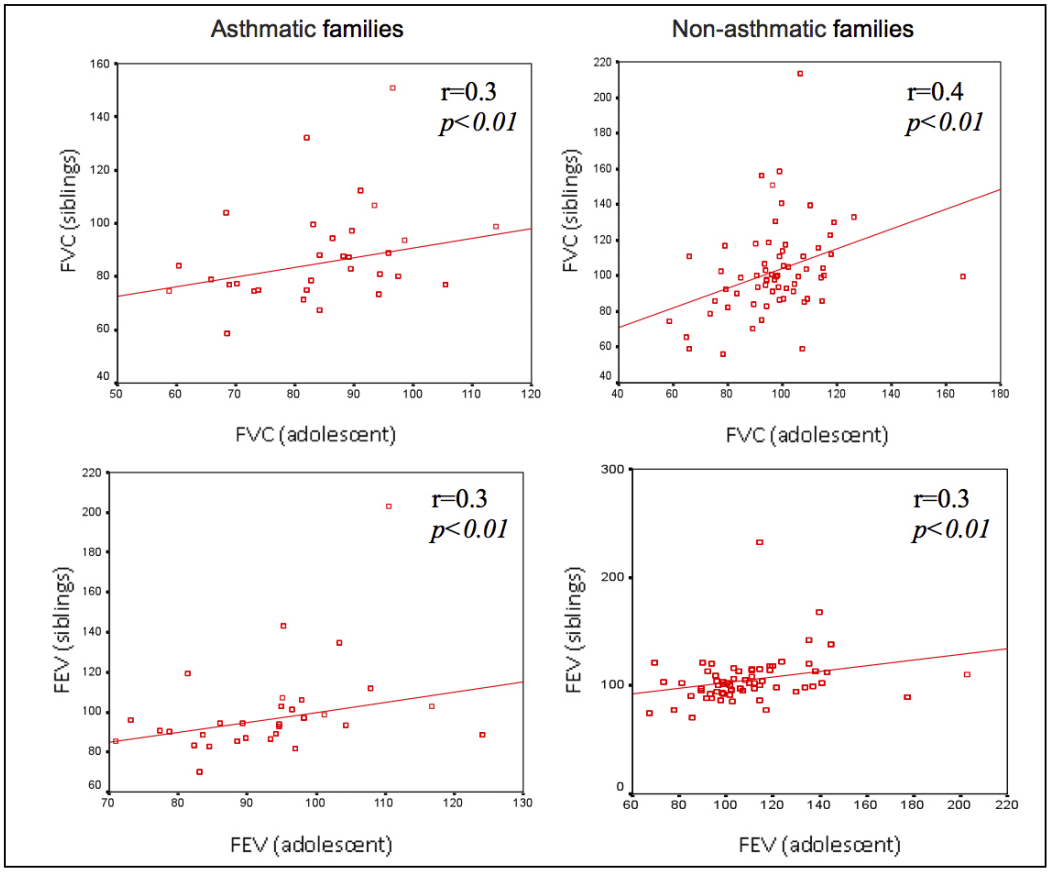
RESULTS

The asthmatic adolescents showed lower mean values of FEV than non-asthmatic ones (95.2 vs. 110.9 $p<0.01$). The same was observed for FVC values (85.5 vs. 100.3, $p<0.01$). Also, the parents of asthmatic adolescents had significantly lower values of lung function than both parents together and mothers of non-asthmatics (84.6 vs. 97.6, $p<0.01$, for FEV and 84.3 vs. 97.9, $p<0.01$, for FVC, and 97.3 vs. 109.7, $p<0.01$, for FEV and 89.5 vs. 105.5, $p<0.01$, for FVC, respectively). Also, siblings of asthmatic adolescents had lower FEV (98.6 vs. 109.4, $p<0.01$) and FVC (85.9 vs. 102.7, $p<0.01$) (Table 1).

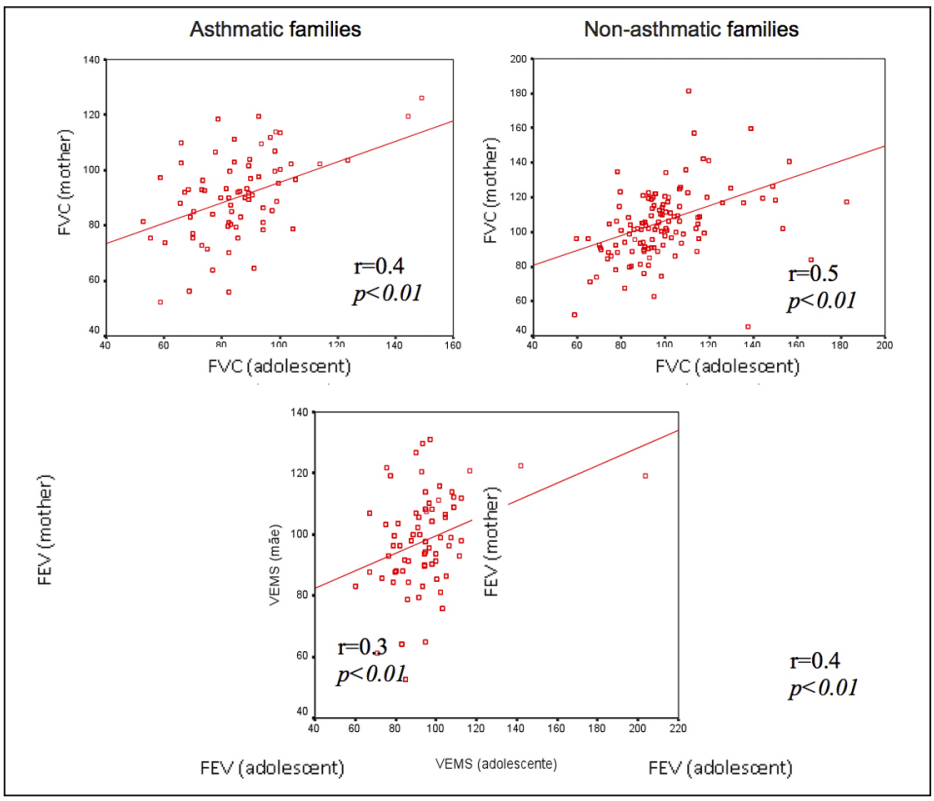
Table 1. Forced vital capacity **(FVC)** and forced expiratory volume **(FEV)** in relatives of asthmatic and non-asthmatic adolescents.

	FEV					***FVC***				
	Relatives of asthmatic **adolescentes**		Relatives of non-asthmatic **adolescents**			Relatives of asthmatic **adolescentes**		Relatives of non-asthmatic **adolescents**		
	N	$\overline{X} \pm SD$	N	$\overline{X} \pm SD$	*p*	N	$\overline{X} \pm SD$	N	$\overline{X} \pm SD$	*p*
Index	99	95.2±12.5	190	110.9±12.2	*<0.01*	99	85.5±10.2	190	100.3±12.4	*<0.01*
Father	77	84.6±15.7	127	97.6±15.8	*<0.01*	77	84.3±14.9	127	97.9±16.0	*<0.01*
Mother	77	97.3±14.2	144	109.7±16.0	*<0.01*	77	89.5±14.3	144	105.5±19.6	*<0.01*
Siblings	26	98.6±14.2	82	109.4±15.7	*<0.01*	26	85.9±14.4	82	102.7±16.6	*<0.01*

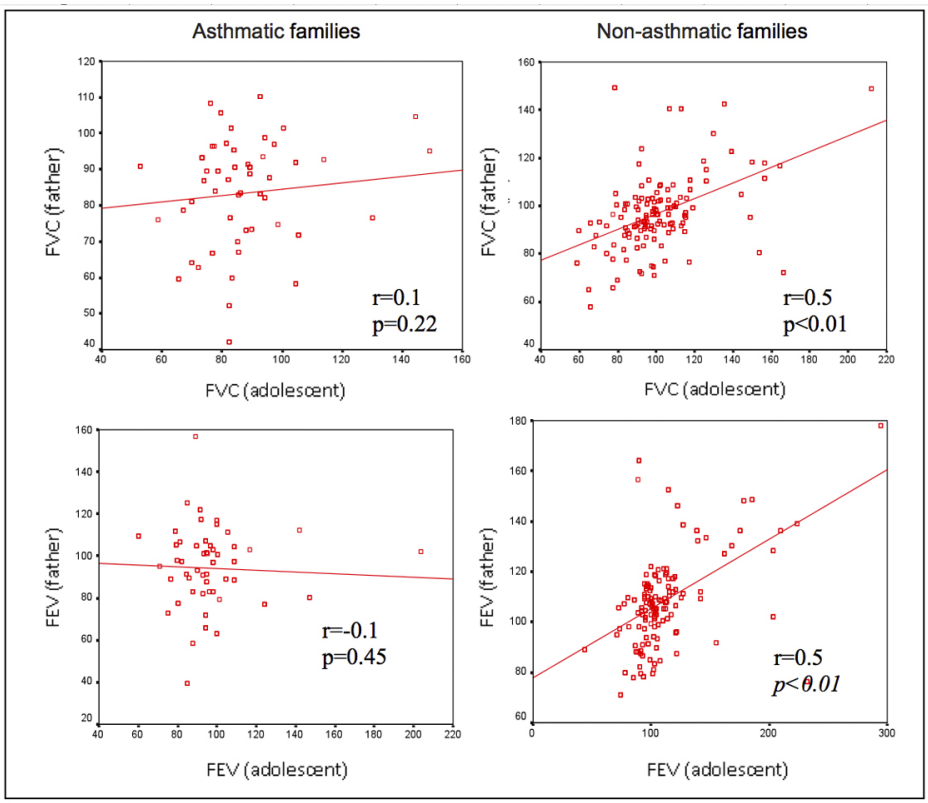
In families with asthmatic adolescents, the FVC values of adolescents were significantly correlated with those of their siblings (r = 0.3) and those of their mothers (r = 0.4). In families without cases of asthma, the FVC values of adolescents were significantly correlated with those of their siblings (r = 0.4), mothers (r = 0.5) and fathers (r = 0.5) and the FEV values were significantly correlated with those of their siblings (r = 0.3), mothers (r = 0.4) and parents together (r = 0.5). In both the families with and without asthmatic cases, there was no correlation between the FEV or FVC values of both parents and those of the mothers.



Figures 1 to 4. FVC and FEV of adolescents and siblings in asthmatic and non-asthmatic families.



Figures 5 to 7. FVC and FEV of adolescents and mothers in asthmatic and non-asthmatic families.



Figures 8 to 11. FVC and FEV of adolescents and parents in asthmatic and non-asthmatic families.

DISCUSSION

The results of the present study showed lower respiratory parameters (FEV and FVC) among adolescents with history of asthma as well as in their first-degree relatives (parents and siblings) regardless of their age, sex and height. On the other hand, the FEV and FVC values of adolescents were positively correlated with those of the different members of the family, in both the families with and without asthmatic cases, except those of adolescents and parents in asthmatic families.

Considering that asthma is a disease with an important genetic component, exacerbated in a complex manner by environmental factors, the magnitude of the relative influence of each factor is difficult to measure (Ober *et al.*, 2011; Burke *et al.*, 2003; Arshad *et al.*, 1993). Since the aggregation of the disease and its indicators has been verified, one is tempted to infer its familial transmission. However, the familial aggregation of this disease does not necessarily imply genetic transmission, because it may result from environmental exposure shared by the various members of the family. The magnitude of the risk associated with "exposure" depends on the methodology used in the research, which raises additional difficulties in generalizing the findings. Nevertheless, several studies have shown that children and adolescents with asthmatic first-degree relatives have a higher risk of getting the disease than those who have no family history of asthma (Bedolla-Barajas *et al.*, 2013; Gu *et al.*, 2012; Lima *et al.*, 2012; Burke *et al.*, 2003; Litonjua *et al.*, 1998; Jenkins *et al.*, 1997; Burrows *et al.*, 1995).

The results of the present study support the conclusions of other studies that have shown the existence of familial aggregation of lung function (Koppelman *et al.*, 2002; Palmer *et al.*, 2001; Chen *et al.*, 1999; Xu *et al.*, 1999; Givelber *et al.*, 1998; Chen *et al.*, 1997; Chen *et al.*, 1996). Considering that the comparisons made in the present study were restricted to the family members with no history of asthma or bronchitis, the aggregation found, even under the medical threshold (i.e. without respiratory disease symptoms), may result from the fact that these members share constitutional characteristics. Therefore, beyond the expression of asthma with obvious physical symptoms, there may be a pattern of deficits among asthmatic relatives. This aggregation can be explained by vertical transmission, since the values of the parameters studied were correlated among family members. This removes the consistency of the hypothesis that the aggregation may result from environmental exposure.

Some studies have reported an increased risk of developing atopic diseases when transmission occurs through the maternal line (Burrows *et al.*, 1995; Dol *et al.*, 1992). However, population-based studies have found no significant differences in the relative risk of transmission through the mother's or father's family line (Burrows *et al.*, 1995; Dol *et al.*, 1992). In the present study, the values of respiratory function of asthmatic adolescents were correlated only with the mother's values, suggesting that the standard functional respiratory deficit is more influenced by the maternal line.

Over recent years, several chromosomal regions have been identified and genetic variants in genes have been associated with the outcome of asthma (De Wan *et al.*, 2012; Koppelman *et al.*, 2002; Heinzmann *et al.*, 2001). However, although a high proportion of this disease could be explained by genetic factors, its development is modulated by environmental factors, whose effects are expressed individually (Thomsen *et al.*, 2010; Skadhauge *et al.*, 1999). Moreover, the increased frequency of asthma recorded in the past decades cannot be explained by the genetic changes that occur too slowly. Rather, it is likely that genetic factors, together with the environment, which has suffered dramatic changes in the last decades, have made a great number of people susceptible to the disease.

The present study does not allow excluding environmental factors as possible causes of the lower respiratory function parameters found in the relatives of asthmatic adolescents (Abdou *et al.*, 2013; Ober *et al.*, 2011). Thus, we may conclude that the familial aggregation found in lung function may result from genetic factors, environmental factors or both. In addition, this aggregation can exist even without translation into obvious symptoms of asthma. If we consider that this deficient pattern can affect in a lesser or greater degree the ability to perform multiple activities, apparently healthy relatives may express the consequences of this disease, even in an almost imperceptible way.

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EVALUATION OF SIX SINGLE NUCLEOTIDE POLYMORPHISMS FOR BOVINE TRACEABILITY IN THE CONTEXT OF THE ARGENTINE-CHINESE BEEF TRADE

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ABSTRACT

Genetic traceability refers to methods associated with the identification of animals and their products through DNA characterization of individuals, breeds or species. To trace breeds, it is necessary to define the breed groups to analyze, and the most appropriate molecular marker set. The selection of genetic markers depends on the gene frequency distribution, the genetic distance among breeds and the presence of private alleles. In this study, we assessed six single nucleotide polymorphisms (SNPs) located in the DGAT1, TG, LEP, GH, FABP4 and GnRHR genes, as potential genetic markers to be included into a panel for genetic traceability for the identification of breed origin associated with the bovine beef trade. The results of the genetic characterization of four of the main Chinese cattle populations and of the principal breeds raised in Argentina and in the world (five *Bos taurus* and two *B. indicus*) suggest that these SNP markers can be successfully used as a part of an effective traceability system for the identification of cattle breed origin in the context of the Chinese meat imports, and in particular in the Argentine-Chinese beef trade.

Key words: breed traceability, genetic traceability, single nucleotide polymorphism, Chinese cattle, Argentine cattle

RESUMEN

La trazabilidad genética, la cual se basa en la identificación de animales y sus productos, permite la identificación individual, racial o de especie. Esta metodología es útil para detectar fraudes y valorizar producciones locales. Para llevar a cabo la trazabilidad es necesario definir los grupos raciales a analizar y el panel de marcadores más apropiados a utilizar. La selección de marcadores depende de la distribución de las frecuencias génicas, de la distancia genética entre las razas y de la presencia de alelos privativos. El objetivo de este trabajo consistió en evaluar seis polimorfismos de nucleótido simple (SNPs) ubicados en los genes *DGAT1*, *TG*, *LEP*, *GH*, *FABP4* y *GnRHR* como posibles marcadores genéticos apropiados para ser incluidos en un panel de trazabilidad para la identificación de la raza de origen en el contexto de la comercialización de carne bovina. Los resultados de la caracterización genética de cuatro de las principales poblaciones bovinas chinas y de las razas más importantes de nuestro país (cinco *Bos taurus* y dos *B. indicus*) sugieren que los marcadores estudiados pueden ser utilizados exitosamente como parte de un sistema de trazabilidad efectivo para identificar el origen de la carne bovina en el contexto de la importación de carne en el mercado chino y en particular en el comercio entre Argentina y China.

Palabras clave: trazabilidad, polimorfismo de nucleótido simple, ganado chino, ganado argentino, comercialización

INTRODUCTION

Nowadays, as a result of technology and globalization, food from all over the world can be found in the market, and meat is not an exception. Moreover, the world meat trade is expected to grow by 16% in 2020 compared with the average of 2008 – 2010, a demand growth that will stem mostly from large economies in Asia, Latin America and the oil exporting countries (OECD - FAO, 2011). In this scenario, China will probably keep its self-sufficiency policy, but due to its potential volumes both in terms of production and consumption, unforeseen events in China could result in a severe impact on the international markets (OECD - FAO, 2011). Besides, consumers from different countries or world regions have singular meat cut preferences that partly generate a trend of countries exchanging meat from the same breeds. Meat trade statistics show particular examples, like those of the US or Canada, which share a large part of their breeds and emerge in the first places as world meat exporters and importers at the same time (USDA, 2013). Consequently, there is a need to distinguish products of different origin due to aspects regarding authenticity (to avoid fraudulent products), quality (diverse productive systems or climates can result in different product merits) and safety (country/regional sanitary status).

During the last several years, consumers have increased their attention to food safety and quality due to the emergence of major diseases (*e.g.,* Bovine spongiform encephalopathy -BSE-, avian influenza, food-borne diseases, etc.) or other health issues (*e.g.,* the dioxin crisis that can generate serious threats to food safety. Furthermore, socio-economic reasons (*e.g.*, changes in food habits towards healthier diets and increased consumption of organic food) have also contributed to the increase in consumer's interests in the origin, production methods, and industrialization processes of animal and plant products (Dalvit *et al.*, 2007). As a consequence, issues associated with the traceability of food products have grown in importance, allowing consumers to consider food origin and processing in their purchasing decisions (Ajmone-Marsan *et al.*, 2004).

It has been seen that consumers from different regions have different tastes and preferences in particular regarding meat cuts. Furthermore, quality attributes are different depending on the market, sometimes oriented to production processes (*e.g.* USDA natural meat), animal welfare (*e.g.* nutrition with GMO-free rations), and/or regulatory barriers (BSE or dioxin control). This results in a differentiation of meat products and creates a need of certification of guaranteed quality and safety of the beef production and the whole supply chain. In this sense, traceability has been a key issue to support the consumer's confidence. In particular, Argentine beef is well known all over the world for its superior quality, supported by the natural production system and the breeds raised (mostly British breeds). Consequently, a breed traceability system could complement the current system to guarantee that quality.

Several identification methods, including ear tags, ruminal bolus, retinal analysis, DNA markers, and tracking devices, have been evaluated to develop traceability systems. These methods allow product traceability at different levels: individual, geographical, breed and/or species (Negrini *et al.*, 2008a; Negrini *et al.*, 2008b; Li *et al.*, 2009). Genetic traceability refers specifically to methods associated with the identification of animals and their products through DNA characterization of individuals, breeds or species. Methods for individual identification are mainly related to food safety, while discrimination between breeds and species is particularly useful to detect commercial frauds and to protect the value of local productions.

At present, microsatellites (STRs) are the markers most widely used for genetic identification and traceability studies (Peelman *et al.*, 1998; Sancristobal-Gaudy *et al.*, 2000; Arana *et al.*, 2002; Vázquez *et al.*, 2004; Herraeza *et al.*, 2005; Dalvit *et al.*, 2006; Orrú *et al.*, 2006; Baldo *et al.*, 2010) due to their high information degree provided by the large number of alleles that can be detected at each locus (Vignal *et al.*, 2002; Dalvit *et al.*, 2008). However, during the last few years, single nucleotide polymorphisms (SNPs) have become popular because they are based on the fundamental unit of genetic variation and are abundant across the genome. Moreover, SNPs have genetic stability, lower rates of genotyping error, and are amenable to automation and high-throughput genotyping technologies (Heaton *et al.*, 2002; Heaton *et al.*, 2005; Mariani *et al.*, 2005; Werner *et al.*, 2004, Karniol *et al.*, 2009; Allen *et al.*, 2010).

The characterization of a universal marker panel for breed traceability is complex because the most appropriate molecular markers for the specific breed groups that need to be assessed must be identified. The selection of markers will therefore depend on the gene frequency distribution, the

genetic distance among breeds, and the presence of private alleles in target populations. To date, two major strategies have been used to characterize genetic traceability systems. One of them involves the use of STRs or SNPs, together with assignment tests (probabilistic strategy; Dalvit *et al.*, 2007; Negrini *et al.*, 2008a; Negrini *et al.*, 2008b), whereas the other one is based on typing specific genes with private alleles (*e.g.,* coat color genes), which eliminates the need for statistical inference (deterministic strategy; Ajmone-Marsan *et al.*, 2004). Both strategies require, however, a preliminary characterization of the genetic structure of the populations under study.

In China, there are four Yellow cattle breeds that possess higher meat production capability than the other Chinese Yellow cattle breeds (Longworth *et al.*, 2001). These four populations represent cattle from the north region and central agricultural region of China. Both *Bos taurus* and *B. indicus* as well as mixed breeds can be found within Chinese native cattle. Traditionally, the so-called Yellow cattle are classified into three groups: humpless, semi-humped, and humped types, coinciding with their distribution from the north to the south of China. In this sense, it has been demonstrated that cattle from the south and southwest have greater *B. indicus* influence (Jia *et al.*, 2007) and that cattle from the northern region are related to European breeds (Sun *et al.*, 2008). In the central agricultural region, cattle are mainly humped or semi-humped, as a result of northern Taurine and southern Zebuine crossbreeding (Sun *et al.*, 2008). These breeds present complex patrilineages (Y-chromosome) and combined matrilineages (mtDNA) between *B. taurus* and *B. indicus*. During the last years, several commercial cattle breeds, including Holstein, Limousine and Simmental, have been introduced to China to improve dairy and beef production.

In Argentina, there are two main breeding areas: the temperate Pampa, where Holstein and British breeds, such as Angus and Hereford, are predominant, and the northeast subtropical region, where Zebuine and Creole cattle are the breeds most commonly raised. These four breeds were selected for the present study because they represent the main beef breeds raised in Argentina. Additionally, Wagyu, a valuable breed with specific meat quality raised in lower scale in Argentina, was included in the study (Rearte, 2007).

Considering the main features of Chinese and Argentine meat production, the aim of this study was to assess six SNPs located in candidate genes for meat quality as potential genetic markers to be included into a traceability panel for the identification of breed origin in the context of bovine beef trade. The candidate genes selected were: AcylCoA-diacylglycerol-acyltransferase 1 (*DGAT1*), Thyroglobulin (*TG*), Leptin (*LEP*), Growth hormone (*GH*), Fatty acid binding protein 4 (*FABP4*), and Gonadotropin releasing hormone receptor *(GnRHR*).

MATERIALS AND METHODS

Sample collection

Meat samples were collected in four Chinese commercial slaughterhouses from 80 individuals classified as Chinese Yellow cattle. One of the slaughterhouses was located in the north region (named herein Ch2) and three in the central agricultural region (named Ch1, Ch3 and Ch4 according to their northeast to southwest geographical location). In addition, blood samples were collected from 243 animals belonging to five populations of *B. taurus* breeds: Angus (AA), Hereford (HE), Holstein (HO), Wagyu (WA) and Argentine Creole (CR), and two *B. indicus*: Brahman (BR) and Nelore (NE). These populations represent the main breeds raised in the world and/or Argentina. In order to take a representative genetic profile of each breed, the samples were collected from several farms that comprise the different genetic lines present in each breed.

DNA extraction

Total DNA was extracted from blood samples using the Wizardâ Genomic DNA purification kit (Promega, Madison, WI, USA) following the manufacturer's instructions. DNA was also extracted from meat samples according to the methods previously reported by Wagner *et al.* (1994) and Giovambattista *et al.* (2001).

SNP genotyping

SNPs were genotyped by pyrosequencing methods, as described by Lirón *et al.* (2010) and Ripoli *et al.* (2011a). SNPs included K232A of *DGAT1*, GH6.1 of *GH*, R4C of *LEP*, I74V of *FABP4*, a T/C silent mutation in the fifth exon of the *GnRHR* gene, and the transition in the *TG* 5´ leader sequence (Grisart *et al.*, 2002; Yao *et al.*, 1996; Liefers *et al.*, 2002; Hoashi *et al.*, 2008; Barendse *et al.*, 2001). Specific

details of the SNPs analyzed are summarized in Table S1. The pyrosequencing assay comprised an initial PCR reaction of the target gene using one of the biotinylated primers. Amplified products were then purified by the streptavidin-coated Sepharose beads capture method to be used as pyrosequencing templates (Ronaghi, 2001). An internal sequencing primer, complementary to the biotinylated strand, was finally used to differentiate the allele variants of the target SNP. Pyrosequencing reactions were run on a PSQ96MA sequencer using the Pyro Gold Reagent Kit (Quiagen GmbH, Hilden, Germany) and analyzed using standard software for pyrosequencing genotyping (Biotage AB, Sweden).

Statistical analysis

Measures of genetic variability: The GENEPOP 4.0 software (Rousset, 2007) was used to calculate the allele frequencies for each SNP locus in all the populations studied. The observed (h_o) and unbiased expected heterozygosity (h_e) for each locus and the average heterozygosity over all loci (H_e) were estimated according to Nei (Nei, 1978), using the ARLEQUIN 3.5 software for population genetic analyses (Schneider *et al.*, 2000). Potential deviations from the Hardy-Weinberg equilibrium (HWE) were estimated by F_{IS} statistics (Weir and Cockerman, 1984) and tested for each locus and population, as well as for all loci, using the exact test included in GENEPOP.

Genetic structure and population differentiation: Genetic structure and genetic differentiation among breeds were assessed through standard Wright's F_{ST} statistics, using the variance-based method of Weir and Cockerham (1984) and with the exact G test (Goudet *et al.*, 1996) for population differentiation. These parameters were estimated using GENEPOP.

Levels of genetic differentiation between populations were described through population pairwise F_{ST} indices and represented graphically using the R-function: pairFstMatrix.r (Schneider *et al.,* 2000). To assess the proportion of genetic variance explained by differences among and within breeds/populations, we initially performed an analysis of molecular variance (AMOVA) for all loci and for each individual locus considering all breeds as a single group. To estimate the proportion of genetic variance explained by individual breeds versus country of origin, we performed a hierarchical AMOVA, in which breeds/populations were grouped by their origin (*i.e.*, European Taurine, Asiatic Taurine, and Zebuine). This analysis was also performed for each locus individually and for all loci. The AMOVA was carried out using ARLEQUIN. To condense the genetic variability revealed by the six SNPs, allele frequencies were used to perform a Principal Components Analysis (PCA) according to Cavalli-Sforza *et al.* (1994) and implemented using the PAST software (Hammer *et al.*, 2001).

Nei´s standard genetic distance ($D_{S;}$ Nei, 1972) was calculated from allele frequencies to perform a cluster analysis using the unweighted pair group method with arithmetic mean (UPGMA, Sneath and Sokal, 1973) algorithm. Confidence for the groupings was estimated through bootstrap resampling of the data using 1000 replications. Genetic distances and trees were computed using the POPULATIONS 1.2.28 software (Langella, 1999). The trees were then visualized using TREEVIEW (Page, 1996).

Assignment test: Assignment tests were performed using simulations of 10000 multilocus genotype data generated from gene frequencies from each of the eleven breeds, assuming Hardy-Weinberg and linkage equilibrium (Paetkau *et al.*, 1995; Liron *et al.*, 2007). This assignment test involves calculating the expected frequency of each individual's genotype in each of the breeds/populations studied and, subsequently, estimating assignment probabilities of each individual to the population where its expected genotype frequency was the highest. Finally, we calculated the percentage of samples correctly assigned to each breed.

RESULTS

Estimates of genetic variability within breeds

Allele frequencies for all breeds are presented in Table 1. The DGAT1-K and FABP4-C variants had higher allele frequencies in Zebuine and Ch3 breeds. In contrast, the DGAT1-A allele was the most abundant variant in Taurine breeds. The FABP4-T and -C alleles exhibited similar incidence in Taurine breeds except HE, which showed a high frequency for FABP4-T. For *GnRHR*, both allele variants had similar frequencies in most of the Taurine breeds, while GnRHR-A was the most common allele in CR, WA, Ch2 and Zebuine breeds. TG-C and GH-C alleles showed relatively high frequencies in all the breeds studied except WA. The LEP-A allele was the most abundant in the British breeds, while the LEP-G allele

showed higher frequencies in all the other breeds.

From a total of 66 locus-population possible combinations, we were able to perform 64 HWE tests. Two locus-population combinations were excluded because of limited polymorphism; one allele variant was fixed for locus *GH* in the BR population and a second allele was almost fixed with *TG* at very high frequency (Minimum Allele Frequency, MAF < 0.013) in the HE population. HWE tests revealed that only five locus-population combinations were statistically significant ($P \leq 0.05$) (Table 2). These deviations corresponded to single-locus differences in HE, CR, WA, Ch3 and Ch1. Non-significant deviations from HWE were observed for the other six breeds analyzed.

Estimates of observed (ho) and unbiased expected (he) heterozygosities for each locus and breed studied are shown in Table 2. The expected heterozygosity ranged from 0.026 for *TG* in the HE breed to 0.508 for *GnRHR* in the Ch1 population and for *FABP4* in the CR, WA, HO, Ch1 and Ch4 breeds/populations. The average heterozygosity (He), also estimated for each population, ranged from 0.261 in NE to 0.455 in Ch2 cattle.

Genetic structure and levels of population differentiation

The F_{ST} index and the exact G test for population differentiation were used to analyze the degree of genetic differentiation among the cattle breeds studied. F_{ST} showed significant differences across all cattle populations (F_{ST} = 0.1530), ranging from 0.056 to 0.260 for each individual locus (Table 2 and Figure S1). The exact G test for population differentiation indicated that differences in allele frequency distributions among populations were highly significantly (exact P value for all loci ≤ 0.0001).

The overall AMOVA, which considered all breeds as a single group, revealed that 15.87% of the genetic variance observed was explained by differences among populations, whereas the other 84.13% was explained by differences among individuals within populations. The hierarchical AMOVA allowed partitioning the genetic variability in different breed groups based on their historical origin. When breeds were grouped in three clusters according to their origin, the AMOVA showed that differences among groups accounted for 8.75% of the total genetic variance, while differences among populations within groups accounted for 9.62%. As with the overall AMOVA, most of the variation (81.63%) was explained by differences among individuals.

The AMOVAs were also performed for each individual locus, considering all breeds as a single group. This analysis revealed a wide range of variation in the proportion of variance explained by differences among populations (5.59 - 25.96%), with the majority of the variation explained by differences among individuals within populations (74.04 - 94.41%, Table 3). The highest percentage of variation among populations was detected for the *GH* gene, with 25.96% of the variation being explained by allele frequencies at this locus. *GnRHR* was the less informative locus regarding population structuring, with only 5.59% of the variation explained by differences among populations.

When populations were grouped according to their European, Asiatic or Zebuine origin, the hierarchical AMOVA also revealed considerable levels of variation within populations (73.31 to 92.56%). However, the genetic variance among groups accounted for up to 19.56% of the genetic diversity, depending on the genetic marker considered (0.72 to 19.56%), whereas the variance among populations within each group explained 4.53 to 24.18% of the total genetic variance.

Allele frequencies were used to generate the D_S for each pair including the 11 cattle populations studied. A distance matrix based on the UPGMA algorithm was used to perform a cluster analysis and assess relationships between cattle breeds. The UPGMA cluster analysis using the D_S was consistent with the historical and geographical origin of population/breeds. The tree revealed the main divergences separating the Asiatic Taurine, European Taurine and Zebuine clusters (Figure 1).

Principal Components Analysis (PCA)

Results from the PCA are reported in Figure 2, which illustrates the first and second Principal components (PCs) for the six SNPs frequency distributions of the populations/breeds analyzed. The first two components cumulatively accounted for 84.54% of the variability in the data. The first PC accounted for 51.24% of the total variance and showed a differentiation pattern between Zebuine breeds (NE and BR) and British breeds (AA and HE). These breeds were mainly differentiated by the allele frequencies at the *DGAT1*, *LEP* and *FABP4* loci and, to less extent, by the *GnRHR* gene frequencies. The second PC explained 33.30% of the variation and clearly distinguished WA from all the other populations. The second PC was explained mainly by differences in allele frequencies at the *GH* and *TG* loci. Although the third PC accounted for 8.53%

of the variance, it provided no information regarding the potential origin of the breeds. PCA results were also consistent with the overall results of the cluster analyses generated using the UPGMA algorithm.

Assignment test

An assignment test was performed using the simulated multilocus genotype data. The results for correct allocation percentage were variable among breeds, ranging from 5.94% in HO to 81.51% in WA. However, for all the breeds except HO, the highest percentage of individual assignment within each breed matched to its own population (Table S2). In general, the highest percentage of wrong allocation corresponded to more closely related breeds. For example, AA was confused with HE and NE was confused with BR. These results are in agreement with the grouping pattern obtained with PCA. An assignment test considering groups of breeds by their origin was calculated and is shown in Table 4.

Table 1. Estimated gene frequencies for DGAT1, GH, LEP, FABP4, GnRHR and TG polymorphisms in Chinese Yellow cattle (named Ch1, Ch3 and Ch4), Angus (AA), Hereford (HE), Holstein (HO), Wagyu (WA) and Argentine Creole (CR), Brahman (BR) and Nelore (NE) breeds/populations. N = sample size.

		FABP4		TG		DGAT1		LEP		GH		GnRHR		N
Breed Origin	Breed	C	T	C	T	A	K	A	G	C	G	A	G	
Great Britain	AA	0.4868	0.5132	0.8448	0.1552	0.8158	0.1842	0.7321	0.2679	0.73	0.27	0.4425	0.5575	29
Great Britain	HE	0.2826	0.7174	0.9872	0.0128	0.9459	0.0541	0.75	0.25	0.85	0.15	0.5438	0.4563	26
Netherlands	HO	0.4444	0.5556	0.7778	0.2222	0.8158	0.1842	0,2857	0,7143	0.85	0.15	0.5179	0.4821	28
Argentina	CR	0.5556	0.4444	0.75	0.25	0.6667	0.3333	0.2812	0.7188	0.92	0.08	0.6923	0.3077	20
Japan	WA	0.55	0.45	0.3235	0.6765	0.5	0.5	0.17	0.83	0.19	0.81	0.6944	0.3056	20
China	Ch1	0.45	0.55	0.7368	0.2632	0.825	0.175	0.2647	0.7353	0.8611	0.1389	0.45	0.55	20
China	Ch2	0.525	0.475	0.775	0.225	0.675	0.325	0.3	0.7	0.5789	0.4211	0.6316	0.3684	20
China	Ch3	0.8611	0.1389	0.8929	0.1071	0.4	0.6	0.225	0.775	0.8333	0.1667	0.4	0.6	20
China	Ch4	0.55	0.45	0.875	0.125	0.725	0.275	0.325	0.675	0.95	0.05	0.575	0.425	20
USA	BR	0.7632	0.2368	0.8929	0.1071	0.2059	0.7941	0.18	0.82	1	0	0.7353	0.2647	20
Brazil	NE	0.8529	0.1471	0.9	0.1	0.3077	0.6923	0.12	0.88	0.91	0.09	0.8636	0.1364	20

Table S1. Details of the SNPs analyzed.

Gene	SNP	Function	Mutation	Association	Authors
DGAT1	CC/GA	Microsomal enzyme that catalyzes the final step of triglyceride synthesis	K232A (eighth exon)	Intramuscular fat content	Ripoli *et al.,* 2011
TG	C/T	Precursor of T3 and T4 in the thyroid gland	C→T (5´ leader sequence)*	Intramuscular fat deposition	Barendse *et al.,* 2001
LEP	C/T	It acts as a lipostatic signal that regulates whole-body energy metabolism	R4C (second exon)	Serum leptin concentration, feed intake, milk yield, body fatness and with marbling scores	Liefers *et al.,* 2002
GH	C/G	It plays a major role in tissue growth, fat metabolism and homeorhesis	L217V (fifth exon)	Carcass composition, marbling and milk production	Yao *et al.,* 1996
FABP4	A/G	Cytoplasmic protein involved in free fatty acid transport and metabolism	I74V (second exon)	Beef flavor and tenderness	Hoashi *et al.,* 2008
GnRHR	T/C	It plays a critical role in sexual differentiation and reproduction	Silent mutation (fifth exon)	Reproduction traits	Liron *et al.,* 2010

Table 2. Observed (ho) and expected (he) heterozygosities, average heterozygosity (He), significant FIS index and FST for six SNPs in Chinese Yellow cattle (named Ch1, Ch3 and Ch4), Angus (AA), Hereford (HE), Holstein (HO), Wagyu (WA) and Argentine Creole (CR), Brahman (BR) and Nelore (NE) breeds/populations.
nd = not determined.

Breed	FABP4			TG			DGAT1			LEP2			GH			GnRHR			H_e
	h_o	h_e	F_{is} (P value)	h_o	h_e	F_{is} (P value)	h_o	h_e	F_{is} (P value)	h_o	h_e	F_{is} (P value)	h_o	h_e	F_{is} (P value)	h_o	h_e	F_{is} (P value)	
AA	0.553	0.506	0.7451	0.207	0.264	0.1193	0.333	0.303	0.6694	0.393	0.399	1	0.267	0.395	0.0501	0.496	0.496	1	0.394
HE	0.304	0.414	0.3012	0.026	0.026	nd	0.000	0.104	0.0006	0.423	0.382	1	0.233	0.259	0.5046	0.413	0.499	0.1789	0.280
BR	0.474	0.371	0.524	0.214	0.198	1	0.294	0.337	0.5388	0.214	0.304	0.347	0.000	0	nd	0.412	0.401	1	0.269
NE	0.294	0.258	1	0.200	0.189	1	0.462	0.443	1	0.118	0.214	0.177	0.111	0.171	0.1829	0.273	0.241	1	0.261
CR	0.556	0.508	1	0.500	0.387	0.5151	0.278	0.451	0.0280	0.312	0.417	0.528	0.167	0.155	1	0.462	0.443	1	0.393
WA	0.500	0.508	1	0.529	0.451	0.6116	0.800	0.513	0.2087	0.222	0.286	0.395	0.381	0.316	1	0.167	0.437	0.0153	0.420
HO	0.444	0.508	0.6579	0.444	0.356	0.5299	0.263	0.309	0.4893	0.500	0.416	0.376	0.300	0.262	1	0.536	0.508	1	0.393
Ch1	0.500	0.508	1	0.421	0.398	1	0.350	0.296	1	0.412	0.401	1	0.167	0.246	0.2697	0.200	0.508	0.0083	0.393
Ch2	0.350	0.512	0.1967	0.450	0.358	0.5280	0.550	0.450	0.6068	0.400	0.431	1	0.526	0.501	1	0.421	0.478	0.6465	0.455
Ch3	0.278	0.246	1	0.071	0.198	0.1107	0.700	0.492	0.0760	0.250	0.358	0.211	0.067	0.287	0.0179	0.300	0.492	0.1553	0.345
Ch4	0.600	0.508	0.6496	0.250	0.224	1	0.450	0.409	1	0.250	0.450	0.113	0.100	0.097	1	0.550	0.501	1	0.365
F_{ST}	0.107			0.159			0.213			0.206			0.260			0.056			

Table S2. Percentage of individual assignment. Chinese Yellow cattle (named Ch1, Ch3 and Ch4), Angus (AA), Hereford (HE), Holstein (HO), Wagyu (WA) and Argentine Creole (CR), Brahman (BR) and Nelore (NE).

	AA	He	Ho	Cr	Ch2	Ch3	Ch1	Ch4	Br	Ne	Wa
AA	39.33	28.20	1.11	2.92	9.00	3.44	5.95	6.52	1.01	0.64	1.91
He	13.37	72.05	2.09	0.94	3.75	0.40	1.57	5.39	0.15	0.25	0.04
Ho	7.99	13.57	5.94	9.21	12.45	6.14	21.47	15.44	1.68	2.84	3.27
Cr	5.31	8.25	3.39	20.55	7.59	8.03	11.48	15.66	7.19	9.85	2.72
Ch2	9.79	5.79	2.30	6.73	31.18	7.86	7.00	6.81	2.65	4.80	15.10
Ch3	4.72	1.01	0.09	2.31	5.58	47.57	3.56	9.25	14.15	8.90	2.87
Ch1	7.52	11.72	5.32	8.40	10.57	6.99	28.25	14.25	1.43	2.14	3.43
Ch4	6.81	15.13	3.63	10.67	4.55	10.41	11.40	24.53	5.05	7.21	0.62
Br	0.80	0.57	0.15	6.35	0.00	13.22	0.93	6.49	44.66	26.83	0.00
Ne	0.42	0.43	0.13	5.56	3.77	10.46	0.48	5.09	22.00	49.60	2.06
Wa	1.25	0.09	0.17	1.60	10.10	1.97	1.34	0.12	0.61	1.24	81.51

Table 3. Percentage of variation for each individual polymorphism (DGAT1, GH, LEP, FABP4, GnRHR and TG) and all loci obtained by AMOVA considering all breeds/populations (Chinese Yellow cattle, Angus, Hereford, Holstein, Wagyu, Argentine Creole, Brahman and Nelore) as a single group.

Source of variation in % of variation	FABP4	DGAT1	GnRHR	TG	LEP	GH	All loci
Among populations	10.06	21.28	5.59	15.88	20.64	25.96	15.87
Within populations	89.93	78.72	94.41	84.12	79.36	74.04	84.13
F_{ST}	0.107(P=< 0.001)	0.213(P=< 0.001)	0.056(P=< 0.001)	0.159(P=< 0.001)	0.206(P=< 0.001)	0.260(P=< 0.001)	0.159(P=< 0.001)

Table 4. Percentage of correct assignment grouped by breed origin: British (Angus and Hereford), HO (Holstein), CR (Argentine Creole), Chinese Yellow cattle, Zebuine (Brahman and Nelore), and WA (Wagyu).

	British	HO	CR	Chinese	Zebuine	WA
British	76.472	1.597	1.928	18.007	1.020	0.975
HO	21.566	5.941	9.212	55.488	4.519	3.274
CR	13.550	3.387	20.550	42.755	17.037	2.721
Chinese	15.620	2.8345	7.026	57.436	11.578	5.506
Zebuine	1.111	0.137	5.957	20.215	71.546	1.032
WA	1.346	0.172	1.597	13.530	1.847	81.508

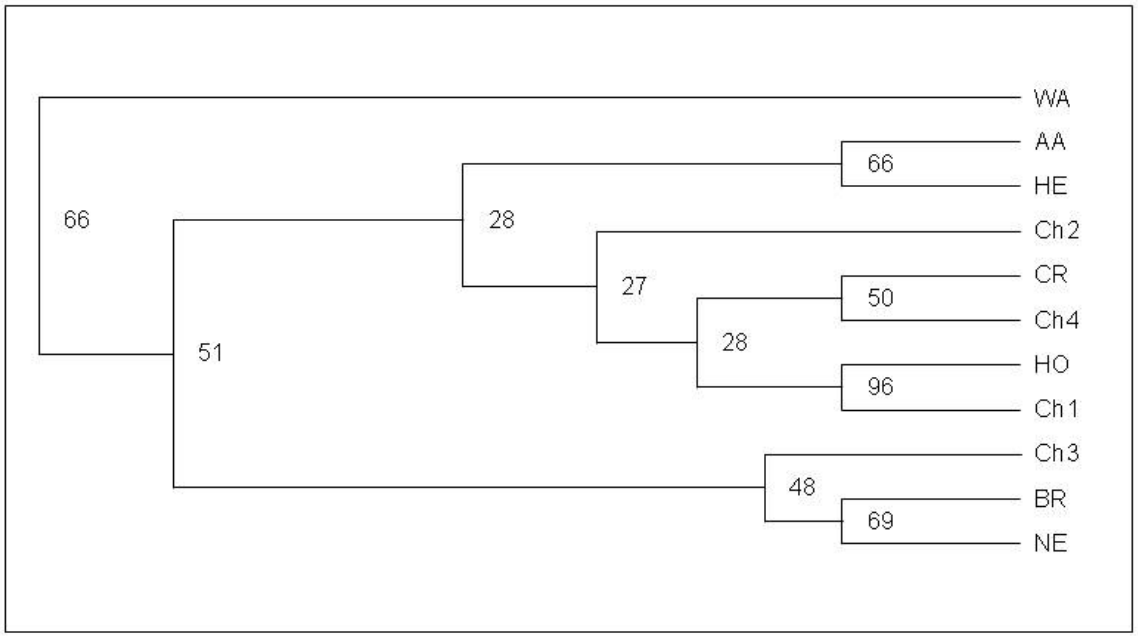
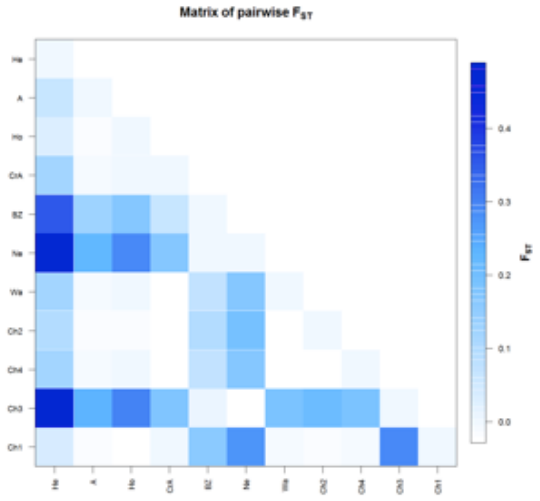
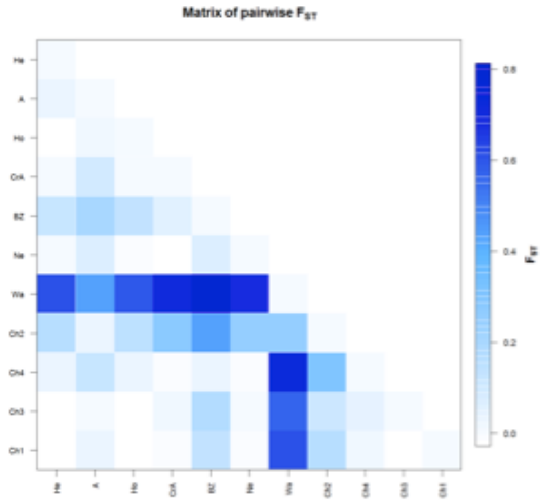


Figure 1. The unweighted pair group method with arithmetic mean (UPGMA) tree was constructed from a matrix of Nei´s standard (Ds) genetic distances using the allele frequencies of the DGAT1, GH, LEP, FABP4, GnRHR and TG polymorphisms in Chinese Yellow cattle (named Ch1, Ch3 and Ch4), Angus (AA), Hereford (HE), Holstein (HO), Wagyu (WA) and Argentine Creole (CR), Brahman (BR) and Nelore (NE).

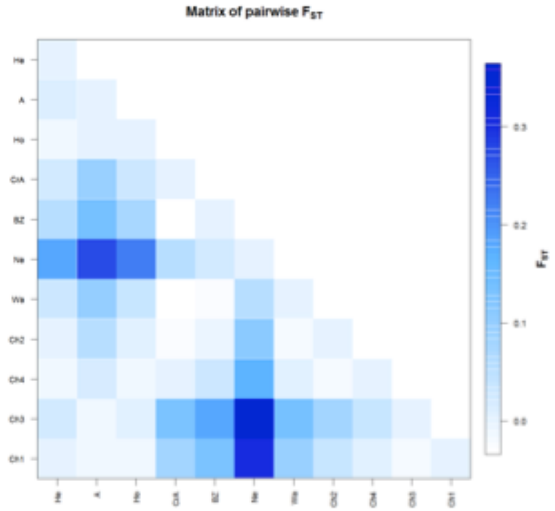
a. FABP4



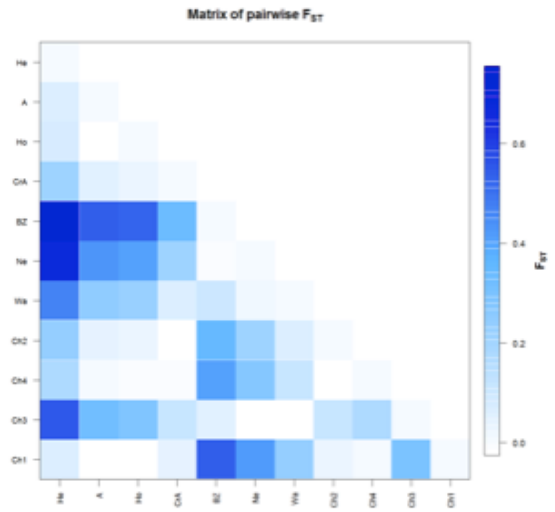
b. GH



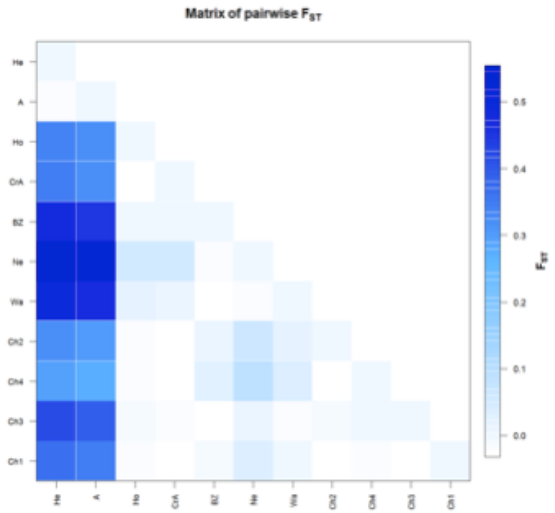
c. GnRHR



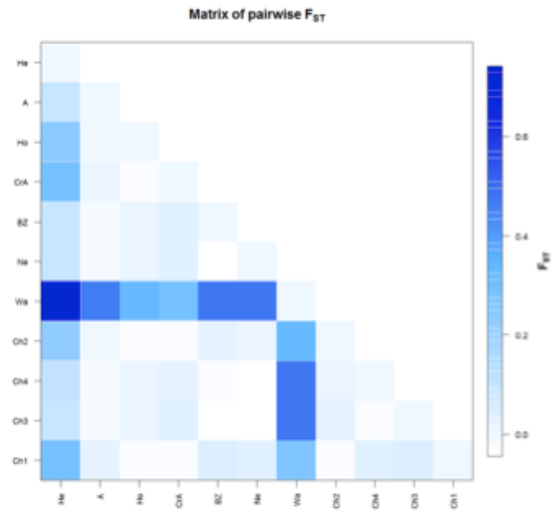
d. DGAT1



e. LEP



f. TG



Supplementary figure S1. Graphic representation of calculated F_{ST} between population pairs using an R-function: pairFstMatrix.r.

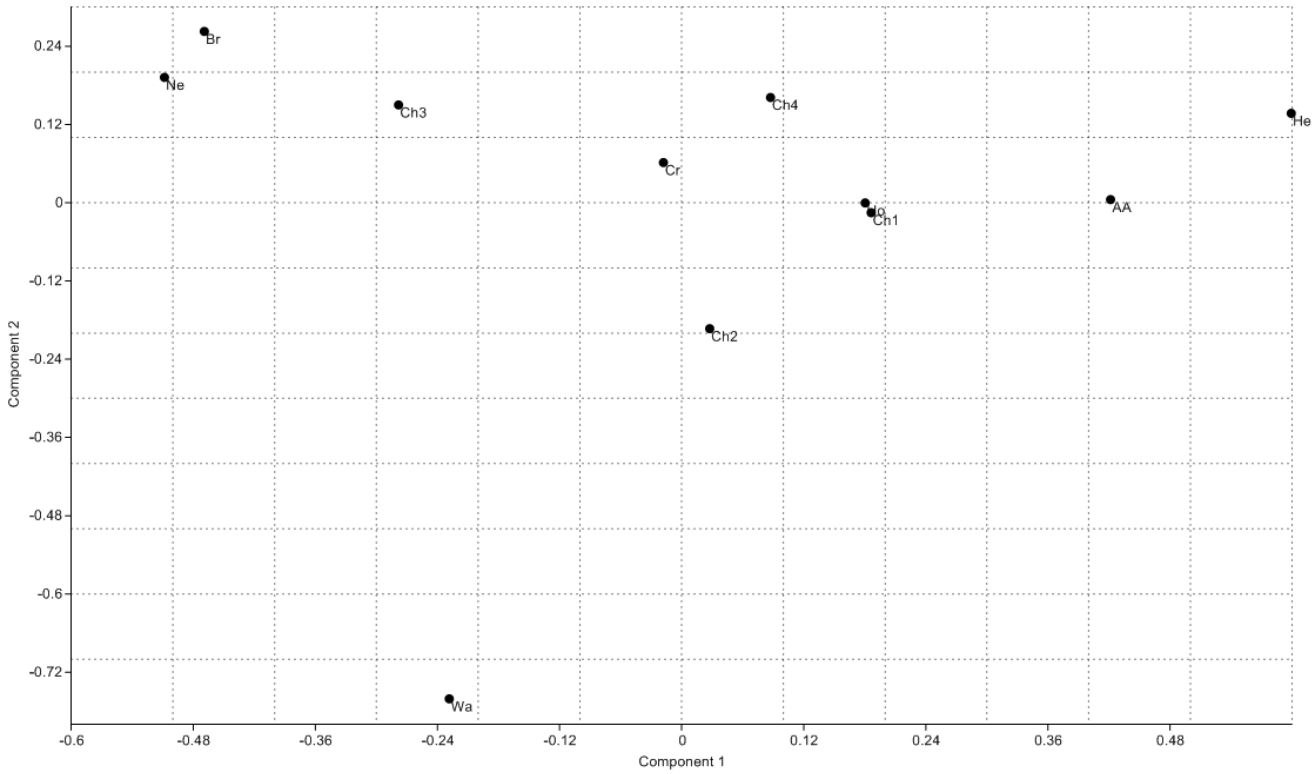


Figure 2. Principal Components Analysis (PCA) of the allele frequencies of the DGAT1, GH, LEP, FABP4, GnRHR and TG polymorphisms in Chinese Yellow cattle (named Ch1, Ch3 and Ch4), Angus (AA), Hereford (HE), Holstein (HO), Wagyu (WA) and Argentine Creole (CR), Brahman (BR) and Nelore (NE) breeds/populations.

DISCUSSION AND CONCLUSIONS

A genetic traceability system is needed to ensure certification of breed origin and beef quality. The first step in the implementation of a genetic traceability system to certify the breed origin of beef products involves the identification of an appropriate set of molecular markers with significant levels of variation that can be explained by group origin, and the genetic characterization of the breed/populations involved in the assessment. In the context of China's beef trading, we assessed the four main beef cattle populations raised in this country and four of the main breeds raised worldwide, to evaluate the feasibility of certifying the origin of breeds or breed products and detecting potential commercial frauds through a genetic traceability system. Furthermore, to certify high quality Argentine beef, mainly from British cattle breeds (such as Argentine AnGus Beef and Argentine Hereford Beef trademarks), HO and Zebuine breeds were included as possible sources of fraud. In addition, beef from WA, a more exotic breed with high marbling grade, was also included because it is produced in Argentina for exportation to premium markets.

The effectiveness of a traceability system will depend greatly on the levels of polymorphisms, allele frequency distributions, and genetic differentiation detected among the breeds under consideration, as well as on the potential presence of private alleles (*i.e.,* breed-specific alleles). In bovines, most differences among breeds are due to differences in allele frequency distributions rather than to the presence of private alleles. As a consequence, most systems require probabilistic methods of traceability, associated with population assignment probabilities, rather than deterministic methods based on population-specific genetic variants. Within the group of SNPs selected in this study, mostly candidate genes for fat content, there were no private alleles, but the gene frequencies showed significant

levels of variation across the breeds studied, accounting for 15.87% of the genetic variance.

The results obtained show that the SNPs analyzed separate WA from the other breeds, and divide the remaining populations into three groups: Taurine, mixed and Zebuine breeds. *GH* and *TG* loci evidenced that the gene frequency profile in WA was notably different from that in the other breeds, as previously observed (Ripoli *et al.*, 2011b), which could be explained by the high level of selection done on WA to increase marbling in this breed (Zembayashi and Lunt, 1995). In the *DGAT1* locus, the allele frequencies evidenced a geographical cline given by the high frequencies for the A allele in European (fixed in HE), Ch1 and Ch4 populations followed by intermediate values in CR, Ch2, WA and lastly by Ch3, NE and BR breeds. These results are also concordant with the degree of Zebu admixture expected within each breed/population analyzed and are in agreement with previous reports (Winter *et al.*, 2002; Ripoli *et al.*, 2006). In the case of the *FABP4* gene, a similar gradient was also observed. The FABP4-T variant showed intermediate frequencies in European and Asiatic Taurine breeds, and lower frequencies in Ch3 and Zebuine breeds. Since Cho *et al.* (2008) reported that the I74V FABP4 polymorphism was associated positively with back fat thickness in Korean cattle, our results could be related to those of Cho *et al.* (2008) because Zebuine breeds are leaner than Taurine ones. The similar frequencies in Ch3, NE and BR may result from the strong influence of Zebu breeds on some populations of the central agricultural region of China, especially on the Ch3 population. Regarding the *LEP* gene, the LEP-G variant exhibited a high frequency in almost all the breeds analyzed, but a low frequency in the British ones. In the *GnRHR* locus, although Zebuine breeds showed higher allele frequencies for the GnRHR-A variant, we could not detect a clear pattern of frequency distribution. Summarizing, *GH* and *TG* gene frequencies seem to be useful to distinguish WA cattle from the other breeds. *DGAT1* and *FABP4* differentiated between Taurine, mixed and Zebuine breeds, as they present a gradient of frequencies, while *LEP* differentiated the British breeds from the rest. All these markers showed F_{ST} values higher than 0.1, which is enough to allow an efficient differentiation of breeds or breed groups. These F_{ST} values were compared with those previously reported using neutral STR markers in the same populations (Liron *et al.*, 2007; Rogberg *et al.*, 2012). The SNP F_{ST} values ranged from 0.107 to 0.26, while the STR F_{ST} values ranged between 0.069 and 0.151. These results evidenced that the SNPs analyzed show greater population structuring than STRs. This fact might be the consequence of selective forces, and supports the hypothesis of the present work that the SNP markers associated with meat quality can be successfully used as a part of an effective traceability system for the identification of cattle breed origin. Regarding *GnRHR*, it seems to be less useful for breed traceability in the Chinese-Argentine beef trade context because it showed a low degree of genetic differentiation among the breeds studied.

Additionally, the tree constructed with UPGMA, using D_S, reflected the same results. The WA breed was located at one end of the tree, and the Zebuine group (NE and BR) and Ch3 population were located at the other end. The British breeds clustered together and the rest of the breeds/populations were placed in an intermediate position between the WA and Zebuine breeds. The tree exhibits a topology consistent with the historical and geographical origin, combined with a selection towards body and/or meat fat content in the population/breeds analyzed. Moreover, the results observed in terms of the PCA also matched with the topology of the tree. In this sense, differences in frequency distribution provide the basis to characterize individual breeds through cluster analysis and PCA.

Finally, an assignment test was performed to validate the effectiveness of the SNPs analyzed to differentiate beef of the main breeds raised in the world and/or Argentina (AA, HE, NE and BR) from premium beef (WA) of those breeds typically raised in China for beef purposes (native breeds and HO). Marker sets previously proposed for breed assignment included more than 11-30 STRs or 25-90 SNPs (Dalvit *et al.*, 2007, 2008; Negrini *et al.*, 2008a,b; Karniol *et al.,* 2009; Baldo *et al.,* 2010). However, although only six SNPs were analyzed in the present work, they evidenced reasonable values of correct allocation. These values are particularly higher (> 70%) for the high quality beef breeds (AA, HE, WA) and lower for the low quality ones (HO, NE and BR). The observed results are in agreement with those obtained with PCA and with the topologies of the tree. The breeds that exhibited extreme values for the first and second PC presented higher percentage of correct allocations. By contrast, breeds with values closer to 0 for the first and second PC showed higher values of wrong allocations. In the case of Argentine high quality beef (AA

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DEVELOPMENT OF TYPING METHODS BASED ON PYROSEQUENCING TECHNOLOGY FOR THE ANALYSIS OF SIX BOVINE GENES RELATED TO MARBLING

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ABSTRACT

Several methods such as PCR-RFLP, OLA, DNA sequencing, PCR-SSCP and ARMS-PCR have been developed to detect the allelic variation at substitutions K232A of *DGAT1*, GH6.1 of *GH*, F279Y of *GHR*, R4C of *LEP*, I74V of *FABP4*, and at the transition in the *TG* 5´ leader sequence. Most of these methods are manual processes and therefore increase the time spent on the assay and limit the number of animals analyzed. Herein, we describe the development of pyrosequencing-based methods for the bovine *DGAT1*, *GH*, *GHR*, *LEP*, *FABP4* and *TG* genes, whose polymorphisms have been associated with variation in carcass composition. This method was validated by analyzing DNA samples belonging to the Aberdeen Angus and Hereford breeds previously typed by PCR-SSCP, PCR-RFLP and/or DNA sequencing. The results obtained showed that, after sequencing or whole-genome association studies (discovery step), the pyrosequencing-based technique seems to be useful to validate (validation step) a particular single nucleotide polymorphism (SNP) in a candidate gene in a previously mapped region in independent populations (with different genotypes and/or production systems). We conclude that pyrosequencing may be useful in high-throughput SNP genotyping of candidate genes in breeds of cattle and other animal species, making it a fast and interesting screening method for population or association studies.

Key words: bovine molecular markers, polymorphism, pyrosequencing, marbling

RESUMEN

Diversos métodos como PCR-RFLP, OLA, secuenciación de ADN, PCR-SSCP y ARMS-PCR han sido desarrollados para detectar las variaciones alélicas presentes en las sustituciones K232A del gen *DGAT1*, GH6.1 del gen *GH*, F279Y de *GHR*, R4C de *LEP*, I74V de *FABP4*, y en la transición detectada en la secuencia 5´ líder del gen *TG*. La mayoría de estos métodos son procesos manuales que consumen mucho tiempo para realizar el ensayo y limitan el número de animales analizados. En el presente trabajo se describe el desarrollo de métodos de pirosecuenciación aplicables a los genes bovinos *DGAT1*, *GH*, *GHR*, *LEP*, *FABP4* y *TG*, cuyos polimorfismos han sido asociados a variaciones en la composición de la carcasa. Este método se validó mediante el análisis de muestras de ADN pertenecientes a las razas Aberdeen Angus y Hereford previamente tipificadas por PCR-SSCP, PCR-RFLP y/o secuenciación de ADN. Los resultados obtenidos evidenciaron que, después de estudios de secuenciación o asociación genómica (etapa de descubrimiento), la pirosecuenciación sería de gran utilidad para validar (etapa de validación) un polimorfismo de nucleótido único (SNP) particular en un gen candidato localizado en una región previamente mapeada en poblaciones independientes (con diferentes genotipos y/o sistemas de producción). Concluimos que los métodos basados en pirosecuenciación pueden ser de gran utilidad en la genotipificación de alto rendimiento de SNPs de genes candidatos en razas bovinas y en otras especies animales, representando un rápido e interesante método de validación para estudios poblacionales o de asociación.

Palabras clave: marcadores moleculares bovinos, polimorfismos, pirosecuenciación, marmoleo

INTRODUCCIÓN

Several single nucleotide polymorphisms (SNPs) have been associated with fat traits in cattle (*Bos taurus*) (de Koning, 2006). Intramuscular fat deposition, or marbling, is an important trait for meat quality since it confers juiciness, flavor and tenderness to beef, hence it contributes directly to the price of beef in international markets.

AcylCoA-diacylglycerol-acyltransferase 1 (DGAT1) is a microsomal enzyme that catalyzes the final step of triglyceride synthesis. A lysine/alanine (K232A) substitution in the protein encoded by the bovine *DGAT1* gene has been shown to be associated with milk fat content (Grisart *et al.*, 2002; Spelman *et al.*, 2002; Winter *et al.*, 2002) and fat deposition in different bovine breeds (Sorensen *et al.*, 2006; Thaller *et al.*, 2003).

Growth Hormone (GH) plays a major role in tissue growth, fat metabolism and homeorhesis (Shingu *et al.*, 2004; Beauchemin *et al.*, 2006; Thomas *et al.*, 2007). The bovine GH gene shows different polymorphisms (Lucy *et al.*, 1991; Zhang *et al.*, 1993; Kirkpatrick *et al.*, 1993), most of which have been associated with differences in carcass composition, marbling and milk production (Lee *et al.*, 1996; Yao *et al.*, 1996; Lechniak *et al.*, 2002, Di Stasio *et al.*, 2005; Curi *et al.*, 2005; Barendse *et al.*, 2006; Thomas *et al.*, 2007). In particular, the GH6.1 polymorphism, also known as AluI RFLP (Yao *et al.*, 1996). is caused by a C to G nucleotide change in exon 5 of the gene, which gives rise to two alleles that are responsible for alternative forms of bovine GH with a Leucine or Valine amino acid residue at position 127.

Growth Hormone Receptor (GHR) has a major role in the regulation of GH action in most tissues. The F279Y polymorphism of the *GHR* gene has been associated with milk traits and carcass quality, especially milk fat content and fat deposition (Blott *et al.*, 2003; Viitala *et al.*, 2006; White *et al.*, 2007). This polymorphism is caused by a T to A replacement in exon 8 and results in a substitution of a Phenylalanine to a Tyrosine residue at position 279 in the mature polypeptide.

Thyroglobulin (*TG*) is the precursor of T3 and T4 thyroid hormones, which have an important role in metabolic regulation and, among other functions, affect lipid metabolism. Barendse *et al.* (2001) reported that the C to T transition in the TG 5´ leader sequence is highly associated with intramuscular fat deposition in long-fed cattle and defines the `2´ (C) and `3´ (T) alleles. Barendse *et al.* (1999, 2004) also found that the TG `3´ allele is more frequent in animals with higher marbling scores.

Leptin (*LEP*) is a protein hormone that plays a major role in whole-body energy metabolism. *LEP* is one of the best physiological markers of body weight, food intake, energy expenditure (Houseknecht *et al.*, 1998; Woods *et al.*, 1998), reproduction (Cunningham *et al.*, 1999; Garcia *et al.*, 2002), and certain immune system functions (Lord *et al.*, 1998). Polymorphisms in the coding regions of the LEP gene in cattle have been associated with serum LEP concentration (Liefers *et al.*, 2003), feed intake (Liefers *et al.*, 2002; Oprzadek *et al.*, 2003), milk yield (Liefers *et al.*, 2002; Buchanan *et al.*, 2003), body fatness (Buchanan *et al.*, 2002; Nkrumah *et al.*, 2004 a, b) and marbling scores (http://ca.igenity.com/igenity_beef1.html). In particular, the C/T polymorphism situated in exon 2 of *LEP* (Liefers *et al.*, 2002), which leads to an Arginine (R) to Cysteine (C) substitution at amino acid 4 (R4C) in the LEP molecule.

The fatty acid binding protein 4 (FABP4) plays a major role in the regulation of lipid and glucose homeostasis through interaction with peroxisome proliferator activated receptors (PPARs), which act as transcription factors in adipocyte differentiation (Mandrup and Lane, 1997). Michal *et al.* (2006) and Lee *et al.* (2010) identified several SNPs in the *FABP4* gene, which have been associated with economically relevant characteristics such as marbling and subcutaneous fat deposition in cattle. In particular, the I74V polymorphism (A to G transition) situated in exon 2, which results in a substitution of an Isoleucine to Valine amino acid residue in the mature polypeptide.

Several methods, such as PCR-RFLP, OLA, DNA sequencing, PCR-SSCP and ARMS-PCR, have been developed to detect the allelic variation at substitutions K232A of *DGAT1*, GH6.1 of *GH*, F279Y of *GHR*, R4C of *LEP*, I74V of *FABP4*, and at the transition in the TG 5´ leader sequence in different breeds (Blott *et al.*, 2003; Buchanan *et al.*, 2002; Cho *et al.*, 2007; Corva *et al.*, 2004; Hoashi *et al.*, 2008; Ripoli *et al.*, 2006; Viitala *et al.*, 2006; Winter *et al.*, 2002; Yao *et al.*, 1996). Most of these methods are manual processes and therefore limit the number of animals analyzed. Improvement of high-throughput methods based on sequencing, pyrosequencing, real-time PCR, TaqMan assay, and microarrays have been developed in recent years. The availability of high-throughput genotyping methods is a valuable tool to

rapidly discover, screen and validate polymorphisms in animal genotyping for association or population studies in animal production. Pyrosequencing™ (Ronaghi *et al.*, 1996, 1998) is a real-time DNA sequencing technique based on the detection of released pyrophosphate (PPi) during DNA synthesis. This technique has been successful for both confirmatory sequencing and *de novo* sequencing (Ahmadian *et al.*, 2000; Ronaghi *et al.*, 1999). After an oligonucleotide is hybridized to a single-stranded DNA template, a cascade of enzymatic reactions starts with the nucleic acid polymerization reaction primed by an internal primer. Each of the four deoxyribonucleotide triphosphates (dNTPs) is then individually added to the reaction mixture, and inorganic PPi is released as a result of nucleotide incorporation by polymerase. Visible light is generated proportionally to the number of incorporated nucleotides (Ronaghi, 2001) detected by a CCD camera and seen as peaks in a pyrogram™.

The aim of this study was to develop a pyrosequencing-based typing method applicable to high- and medium-throughput genotyping of the K232A, GH6.1, F279Y, R4C, and I74V substitutions in the *DGAT1*, *GH*, *GHR*, *LEP*, and *FABP4* genes respectively and at the transition in the TG 5´ leader sequence in different cattle breeds. We applied this method to screen these polymorphisms in a sample of individuals belonging to the Hereford and Aberdeen Angus cattle breeds**,** previously genotyped by direct sequencing, PCR-RFLP and/or PCR-SSCP.

MATERIALS AND METHODS

Sample collection and DNA extraction

Blood samples were collected from 25 Hereford and 25 Aberdeen Angus cattle. Total DNA was extracted from blood samples using the DNAzolâ reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

Polymorphism detection assays

DGAT1 analysis by PCR-SSCP: The K232A polymorphism was analyzed by PCR-SSCP and DNA sequencing as described in Ripoli *et al.* (2006).

GH analysis by PCR-RFLP: The GH6.1 polymorphism was analyzed by PCR-RFLP as described in Yao *et al.* (1996).

GHR analysis by PCR-sequencing: The F279Y polymorphism was analyzed by PCR-DNA sequencing using the primers described in Blott *et al.* (2003) for the PCR (see below), and then sequenced in a MegaBACE 1000 automatic sequencer (GE Healthcare, USA).

TG analysis by PCR-RFLP: The C to T transition in the TG 5´ leader sequence was analyzed by PCR-RFLP as described in Barendse *et al.* (2001).

LEP analysis by PCR-RFLP: The R4C polymorphism was analyzed by PCR-RFLP according to Liefers *et al.* (2002).

FABP4 analysis by PCR-sequencing: The I74V polymorphism was analyzed by PCR-DNA sequencing using primers specially designed for the PCR (see below), and then sequenced in a MegaBACE 1000 automatic sequencer (GE Healthcare).

Pyrosequencing analysis of polymorphisms

a) Preparation of PCR products

DGAT1: A 176 bp fragment of from the DGAT1 gene spanning the K232A substitution (exon 8) was amplified as in Ripoli *et al.* (2006). The forward primer was biotinylated for the subsequent purification step. The internal sequencing primer, complementary to the forward strand, was designed using Pyrosequencing Primer SNP Design 1.01 software (http://www.pyrosequencing.com) (Table 1). This primer is located upstream the SNP selected to differentiate the A and K alleles. The PCR was performed separately with 2 µl DNA in a 25 µl reaction mixture containing 1X PCR buffer (Invitrogen), 0.15 µM for each primer, 200 mM each dNTP, 2 mM MgCl2 and 0.5 units of Taq polymerase (Invitrogen), in a Maxygene Gradient thermocycler (MJ Research, Boston, MA, USA - Bio-Rad Laboratories Inc.). PCR conditions consisted of 45 cycles of 94°C for 45s, 63°C for 45s and 72°C for 45s, plus a final extension at 72°C for 10 min.

GH: A 259 bp fragment of exon 5, including the GH6.1 polymorphism, was amplified by an adaptation of the method undertaken by Schlee *et al.* (1994). The reward primer was biotinylated for the subsequent purification step and the internal sequencing primer was designed complementary to the reward strand (Table 1). This primer is located upstream the SNP selected to differentiate the C and G alleles. The PCR was performed separately with 2 µl DNA in a 25 µl reaction mixture containing 1X PCR buffer (Invitrogen), 0.15 µM for each primer, 200 mM each dNTP, 2 mM MgCl2 and 0.5 units of Taq polymerase (Invitrogen), in a Maxygene Gradient thermocycler (MJ Research - Bio-Rad Laboratories Inc.). PCR conditions consisted of 45 cycles of 94°C for 45s, 63°C for 45s and

72ºC for 45s, plus a final extension at 72ºC for 10 min.

GHR: A 342-bp fragment of the GHR gene of exon 8, including the F279Y substitution, was also amplified according to Blott *et al.* (2003). The forward primer was biotinylated for the subsequent purification step. The internal sequencing primer was designed complementary to the forward strand (Table 1). This primer is located upstream the SNPs selected to differentiate the A and T alleles. The PCR was performed separately with 2 µl DNA in a 25 µl reaction mixture containing 1X PCR buffer (Invitrogen), 0.15 µM for each primer, 200 mM each dNTP, 2 mM MgCl2 and 0.5 units of Taq polymerase (Invitrogen), in a Maxygene Gradient thermocycler (MJ Research - Bio-Rad Laboratories Inc.). PCR conditions consisted of 45 cycles of 94ºC for 45s, 63ºC for 45s and 72ºC for 45s, plus a final extension at 72ºC for 10 min.

TG: A 266 bp fragment spanning part of the TG 5´ leader sequence that included the C/T transition was amplified with specially designed primers. The reward primer was biotinylated for the subsequent purification step, and the internal sequencing primer was designed complementary to the reward strand (Table 1). This primer is located upstream the SNP selected to differentiate the "2" and "3" alleles. The PCR was performed separately with 2 µl DNA in a 25 µl reaction mixture containing 1X PCR buffer (Invitrogen), 0.15 µM for each primer, 200 mM each dNTP, 2 mM MgCl2 and 0.5 units of Taq polymerase (Invitrogen), in a Maxygene Gradient thermocycler (MJ Research - Bio-Rad Laboratories Inc.). PCR conditions consisted of 45 cycles of 94ºC for 45s, 58ºC for 45s and 72ºC for 45s, plus a final extension at 72ºC for 10 min.

LEP: A 269-bp fragment of exon 2, including the R4C mutation, was amplified with primers specially designed. The forward primer was biotinylated for the subsequent purification step and the internal sequencing primer was designed complementary to the forward strand (Table 1). This primer is located upstream the SNP selected to differentiate the C and T alleles. The PCR was performed separately with 2 µl DNA in a 25 µl reaction mixture containing 1X PCR buffer (Invitrogen), 0.15 µM for each primer, 200 mM each dNTP, 2 mM MgCl2 and 0.5 units of Taq polymerase (Invitrogen), in a Maxygene Gradient thermocycler (MJ Research - Bio-Rad Laboratories Inc.). PCR conditions consisted of 45 cycles of 94ºC for 45s, 60ºC for 45s and 72ºC for 45s, plus a final extension at 72ºC for 10 min.

FABP4: A 295-bp fragment of exon 2 including the I74V polymorphism was amplified using primers specially designed. The forward primer was biotinylated for the subsequent purification step and the internal sequencing primer was designed complementary to the forward strand (Table 1). This primer is located upstream the SNP selected to differentiate the A and G alleles. The PCR was performed separately with 2 µl DNA in a 25 µl reaction mixture containing 1X PCR buffer (Invitrogen), 0.15 µM for each primer, 200 mM each dNTP, 2 mM MgCl2 and 0.5 units of Taq polymerase (Invitrogen), in a Maxygene Gradient thermocycler (MJ Research - Bio-Rad Laboratories Inc.). PCR conditions consisted of 45 cycles of 94ºC for 45s, 63ºC for 45s and 72ºC for 45s, plus a final extension at 72ºC for 10 min.

b) Pyrosequencing

After each PCR template generation, the products were purified by capturing the biotinylated strands with streptavidin-coated Sepharose beads (Streptvidin SepharoseTM High Performance, GE Healthcare). This immobilized biotinylated strands were used as pyrosequencing template (Ronaghi *et al.* 1998; Ronaghi, 2001). Pyrosequencing was carried out with the internal sequencing primer diluted to 0.3 µM in the annealing buffer provided by the supplier, using the Pyro Gold Reagent Kit (Biotage, AB, Uppsala, Sweden). A PyroMark Prep Workstation (Biotage AB) was used for all steps other than bead addition and transfer. Samples were run on a PSQTM96 System instrument, and outgoing results were analyzed using pyrosequencing software (Biotage AB).

Calculation of allele frequencies

The ARLEQUIN 2.0 software package (Schneider *et al.* 2000) was used to calculate the allele frequencies for each locus in each population studied.

Table 1. Oligonucleotide primers used in this study.

Gene	Primer type	Sequence (5´to 3´)	Region
DGAT1	Forward	CTTGCTCGTAGCTTTGGCAGG	Exon 8
	Reverse	CGAAGAGGAAGTAGTAGAGATC	
	Internal	AGCTCCCCCGTTG	
GH	Forward	TAGGGGAGGGTGGAAAATGGA	Exon5
	Reverse	GGCACTTCATGACCCTCAGGT	
	Internal	CCCTTGGCAGGAG	
GHR	Forward	GTGGCTATCAAGTGAAATCATTGAC	Exon 8
	Reverse	ACTGGGTTGATGAAACACTTCACTC	
	Internal	TAGAAAATATGAGTAAA	
LEP	Forward	TAACGGAGCACGTGGGTGT	Exon 2
	Reverse	CGGTTCTACCTCGTCTCCCA	
	Internal	TGTCATCCTGGACCTTGC	
TG	Forward	CTTTGGCCTTTACCCCTGAAG	UTR 5´
	Reverse	CCAGGGACGAATGTGTGTGA	
	Internal	CTGGGTTGGGAAGAT	
FABP4	Forward	TCATCAGTTTGAATGGGGGT	Exon 2

RESULTS

Pyrosequencing allowed us to quickly detect all the SNPs analyzed. This method allowed the detection of homozygous and heterozygous genotypes, and the pyrograms obtained for each genotype were coincident with those predicted by the software (PSQ 96 MA 2.1.1.) The method was validated by genotyping 33 DNA samples from Hereford and Aberdeen Angus cattle previously analyzed by direct sequencing, PCR-RFLP and/or PCR-SSCP.

The allele frequencies for the breeds studied are shown in Table 2. Alleles FABP4 A, LEP T, TG 2, DGAT1 A and GH C were the most common variants in the cattle breeds analyzed. Noteworthy, DGAT1 A variants were fixed in Hereford. The exception was *GHR*, where allele GHR A was the most abundant variant in Aberdeen Angus and the least abundant in Hereford. These results are in agreement with data previously reported (Ripoli *et al.*, 2006, 2011).

Table 2. Estimated gene frequencies (in percentage) for six SNPs analyzed in Aberdeen Angus (AA) and Hereford (HE) cattle breeds.

Breed	DGTA1		FABP4		LEP		TG		GH		GHR	
	A	K	A	G	C	T	2	3	C	G	A	T
AA	77.08	22.92	60.00	40.00	22.92	77.08	78.00	22.00	80.00	20.00	86.00	14.00
HE	100.00	-	72.00	28.00	25.00	75.00	98.70	1.30	85.00	15.00	12.50	87.50

DISCUSSION

Multiple SNPs, such as K232A, GH6.1, F279Y, R4C, I74V and TG 5´, have been associated with economically important traits. It is thus necessary to have rapid and efficient SNP evaluation techniques in order to validate these SNPs in independent populations. As a first approach to screen polymorphisms simultaneously in a large number of individuals, SSCP typing is a very useful tool. However, since it is highly temperature- and ion concentration-dependent, electrophoresis reproducibility is a relevant point to be considered. On the other hand, PCR-RFLP is also a useful tool for genotyping, but it is complicated when one should genotype a large number of animals; also, this technique could generate results of false heterozygotes as a consequence of partial digestion. In the case of direct sequencing, the process can also become complex and time-consuming when working with a large number of individuals. In contrast, pyrosequencing, as an automatic sequencing method, is easy to standardize, and furthermore, its throughput is 96 samples in approximately 20 minutes (Ronaghi, 2001; Wittwer *et al.*, 1997). In addition, this technique sequences the flanking regions of the mutation analyzed, confirming the genotyped region and avoiding false heterozygotes.

During the last years, high-throughput and new-generation technologies (microarrays, whole-genome sequences, etc.) have grown exponentially. These techniques allow analyzing several polymorphisms simultaneously, with a very low cost. The pyrosequencing-based method developed in the present work is more expensive than the methods mentioned above (about 1 U$S each SNP). However, this method is useful and the total cost per experiment is more accessible when, after sequencing or whole genome association studies (discovery step), it is necessary to validate a particular SNP in a candidate gene in a previously mapped region in independent (validation step) populations. Also, it is a useful and less expensive when it is necessary to validate a SNP previously associated with a characteristic of production in different genotypes and production systems.

Pyrosequencing is more efficient and faster than direct sequencing, RFLP or SSCP analysis. SNP analysis in large population studies is highly improved due to the reduction in the amount of reagents used, the automation in outcome acquisition and result interpretation. This could aid in the rapid and efficient analysis of SNPs in many genes associated with economic traits in cattle.

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EMBRYOGENIC CALLUS INDUCTION ON THE SCUTELLUM AND REGENERATION OF PLANTS AS BASIS FOR GENETIC TRANSFORMATION OF SPRING WHEAT (*TRITICUM AESTIVUM* L.) CULTIVARS FROM ARGENTINA

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ABSTRACT

An effective tissue culture is an indispensable basis for the production of transgenic plants. In this study, we investigated the induction of embryogenic callus production from the scutellum of spring wheat and subsequent regeneration of plants. Twenty-two Argentine spring wheat varieties from three breeders were screened to select promising genotypes for the optimization of the procedure. Experimental variables were the developmental stage of the immature embryos and the concentration of the growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D). Two developmental stages, termed H and W, respectively (according to He *et al.*, 1986), were selected, and their performance in culture media containing high and low concentrations of 2,4-D was examined. Parameters for the screening were: callus induction, callus proliferation on the scutellum, precocious germination of the immature embryo (as negative trait), regeneration and shoot formation and total efficiency of the *in vitro* culture. The best results were achieved with a combination of developmental stage H and a low concentration of 2,4-D. Scutellar callus induction and callus proliferation on the scutellum surface were positively correlated. However, scutellar callus induction, regeneration and number of shoots were independent of each other. Considerable genotype differences were found in the suitability of the varieties investigated for spring wheat propagation via *in vitro* culture. The cultivar *Klein Brujo* exhibited the highest overall culture efficiency (84.4 %) related to the number of immature embryos used and the number of plants regenerated from one embryo (9.5).

Key words: *In vitro* culture, immature embryos, embryogenic scutellar callus, callus proliferation

RESUMEN

La base indispensable para la producción de plantas transgénicas es un eficaz cultivo de tejidos. En este estudio se investigó la inducción de callo embriogénico y su posterior regeneración en trigo. Se analizaron veintidós variedades argentinas de trigo de primavera de tres criadores, para seleccionar los genotipos adecuados para la optimización del procedimiento. Las variables experimentales fueron la etapa de desarrollo de embriones inmaduros y la concentración de ácido 2,4-diclorofenoxiacético (2,4-D), un regulador del crecimiento. Se seleccionaron dos etapas del desarrollo denominadas H y W, respectivamente (de acuerdo a He *et al.*, 1986) y se examinaron sus comportamientos en los medios de cultivo con concentraciones altas y bajas de 2,4-D. Los parámetros del análisis fueron: inducción de callo, proliferación de callo escutelar, germinación precoz del embrión (rasgo negativo), regeneración y formación de plántulas y eficiencia del cultivo *in vitro*. Los mejores resultados se obtuvieron con una combinación de la etapa de desarrollo H y una concentración baja de 2,4-D. Hubo una correlación positiva entre la inducción de callo y su proliferación escutelar. Sin embargo, la inducción de callo, la regeneración y el número de plántulas fueron independientes entre sí. Las variedades de trigo investigadas mostraron considerables diferencias genotípicas en su capacidad de propagación en cultivo *in vitro*. La variedad *Klein Brujo* exhibió la eficiencia más alta de cultivo (84,4%), en relación con el número de embriones utilizados y de plantas regeneradas (9,5) a partir de un embrión.

Palabras clave: Cultivo *in vitro*, embriones inmaduros, callo embriogénico escutelar, proliferación de callo

INTRODUCTION

Wheat is one of the world's major food crops. Unfortunately, stable transformation efficiency is rather erratic and not very high. For successful biotechnological work with wheat, methodological improvements, such as an efficient system with a high potential of regeneration, are required. Although *in vitro* techniques have been partly established for wheat, many of the factors that affect the development of embryogenic callus and subsequently regulate plant regeneration are not yet well understood. As compared to model genotypes like Florida and Bobwhite (Rasco-Gaunt *et al.,* 2001), high-yield cultivars of wheat perform poorly in both processes (León *et al.,* 2006). As starting material for tissue culture, wheat offers only a few sources of explants that are suitable for regeneration in tissue culture (Sparks and Jones, 2009), e.g. immature zygotic embryos and immature inflorescences. The scutellum surface of immature zygotic embryos is the target most commonly used for wheat genetic transformation with particle bombardment or gene transfer by *Agrobacterium* (Sparks and Jones, 2009). Calli proliferating on the scutellum surface are especially useful for biolistic gene transfer experiments (Viertel *et al.,* 1998). Callus formation capacity and plant regeneration depend not only on the type and age of the explants (Özgen *et al.,* 1998), but also on the genotype (Carman *et al.*, 1987; Fennell *et al.,* 1996; Özgen *et al.,* 1998; Dağüstü, 2008), the culture medium (Carman *et al.*, 1987; Fennell *et al.,* 1996; Barro *et al.,* 1999; Rasco-Gaunt *et al.,* 2001), the plant growth regulators (PGRs) and carbon resource concentrations (Mathias, 1990; Rasco-Gaunt *et al.,* 2001; Almouslem *et al.,* 2005) and the culture conditions (He *et al.,* 1988). The developmental stage of immature zygotic embryos plays an important role in the induction of embryogenic callus (Sears and Deckard, 1982; Maddock *et al.,* 1983; He *et al.,* 1988; Vasil, 1987; Özgen *et al.,* 1998; Rasco-Gaunt *et al.,* 2001). Very young or very old immature embryos usually do not form calli or do so only with low efficiency. Particularly with increasing age, immature embryos tend to germinate precociously (He *et al.,* 1988), which is problematic for the subsequent cultivation. Development of a standardized protocol is influenced by the genotype, the age of the immature embryos and the supply of PGRs to the medium. Addition of the artificial auxin 2,4-dichlorophenoxyacetic acid (2,4-D) is indispensable for the induction of scutellar callus (Scott *et al.,* 1990). Concentrations between 1 (Sears and Deckard, 1982; Dağüstü, 2008) and 2 mg/l 2,4-D (He *et al.,* 1988; Redway *et al.,* 1990; Özgen *et al.,* 1998; Varshney and Altpeter, 2001) have been used for callus induction.

The objective of this work was to improve the vegetative propagation of wheat plants from immature embryos as a basis for genetic transformation. To this end, two steps were investigated in detail: the induction and propagation of scutellar calli and the regeneration of wheat plants from those calli. For callus induction and propagation, in addition to different genotypes, two developmental stages of immature zygotic embryos, and two concentrations of 2,4-D were used. The combination of both variables resulted in four different culture conditions. For a reliable statistical analysis, 22 commercial wheat genotypes from three different breeders were used. The varieties investigated are grown in Argentina, which is one of the main wheat exporting countries (FAO 2012).

MATERIALS AND METHODS

Seeds of 22 Argentine spring wheat cultivars were kindly supplied by three breeders from Buenos Aires province: José Buck S.A. (José Buck), Criadores Klein S.A. (Criadores Klein) and Instituto Nacional de Tecnología Agropecuaria (INTA) (Table 1). Donor plants were grown from these seeds in the greenhouse at 20°C and additional illumination was provided during the winter by mercury discharge lamps (180 µmol $m^{-2}s^{-1}$) to maintain a 16-h photoperiod. The substrate was 70 % compost and 30 % clay.

The developmental stage of immature zygotic embryos was determined from morphological features of the grains according to the Zadoks scale (Zadoks *et al.,* 1974). Grains were surface-sterilized in 25 % (v/v) commercial bleach [10.5 % (v/v) NaOCl; 0.3 % (w/v) Na_2CO_3; 10 % (w/v) NaCl; 0.5 % (w/v) NaOH] for 15 min, followed by four washes with sterile distilled water. Immature embryos were aseptically excised under a stereomicroscope in a laminar flow hood and transferred to sterilized callus induction medium. Two developmental stages of immature zygotic embryos (H and W) were identified by two traits: scutellum and axis of the embryo (Table 2). Developmental stage H corresponded to stage II, whereas stage W corresponded to stage IV, as characterized by He *et al.* (1986) (Fig. 1).

ML3 basal medium (Viertel and Hess, 1996), consisting of L3 medium (Jähne *et al.,* 1991) supplemented with 3 %

(w/v) maltose and two concentrations of 2,4-D (1 or 2 mg/l, respectively), was used for induction and maintenance of the calli (callus induction medium). The medium was solidified with 0.2 % Gelrite. The combination of the two developmental stages of immature embryos and two concentrations of 2,4-D resulted in four culture conditions (A, B, C and D) (Table 2). Twenty-five immature zygotic embryos of each variety and treatment were cultured in petri-dishes (90 x 15 mm) with the scutellum up and the embryo axis kept in contact with the solidified callus induction medium (30 ml). This procedure was repeated five times. After three weeks of incubation at 25°C in total darkness, embryogenic scutellar callus formation, callus proliferation on the scutellum and precocious germination were evaluated in all embryos, under a stereomicroscope.

For regeneration of plants, embryogenic calli were transferred to petri dishes containing modified MSB medium (Viertel and Hess, 1996), consisting of MS salts and B5 vitamins (Gamborg *et al.*, 1968), supplemented with 0.5 mg/l 6-benzylaminopurine (BA) and 0.05 mg/l 1-naphthalene acetic acid (NAA) (Ahuja *et al.,* 1982) and solidified with 0.2 % Gelrite.

Cultivation was at 25°C under 16/8h light/dark periods (ca. 54 µmol $m^{-2}s^{-1}$). Plant regeneration capacity and number of regenerated plants were evaluated after 3-4 weeks under a stereomicroscope. To stimulate root growth, plants were transferred to the root-promoting 190-2 medium, as described by Viertel and Hess (1996) without plant growth regulators, supplemented with 3 % (w/v) sucrose, 1 % (w/v) activated charcoal and 0.2 % Gelrite instead of agar.

Plants with well-developed root systems were transferred into autoclaved soil (see above). After acclimation in a culture room, cultivation was continued in the greenhouse (Viertel and Hess, 1996).

Data were analyzed by a chi-square test of independence (contingency table analysis), determination of variance analysis, standard deviation and correlation. If necessary, the *post hoc* test was carried out between the samples from the four culture conditions (A vs. B; C vs. D; A vs. C and B vs. D). Subsequently, the significance calculated was compared with the manually corrected value (Bonferroni-Correction according to Holm). The statistical software package used was SPSS 13.0 (SPSS 2004).

Table 1. Plant breeders and cultivars of the 22 Argentine spring wheat (*Triticum aestivum* L.) genotypes.

Cultivar	Plant breeder
Buck Charrua (BCh)	José Buck
Buck Poncho (BP)	
Klein Cobre (KC)	Criadores Klein
Klein Pegaso (KP)	
Klein Cacique (KCa)	
Klein D. Enrique (KDE)	
Klein Brujo (KB)	
Klein Escorpión (KE)	
Klein Volcán (KV)	
Klein Dragón (KD)	
Klein Estrella (KEs)	
Prointa Oasis (PO)	INTA
Prointa Elite (PE)	
Don Ernesto Inta (DEI)	
Prointa Imperial (PI)	
Prointa Alazán (PA)	
Prointa Granar (PG)	
Prointa Federal (PF)	
Inta Huenpan (IH)	
Prointa Puntal (PP)	
Prointa B. Remodon (PBR)	
Prointa Cauquén (PC)	

Table 2. Experimental treatments. The combinations of the developmental stages of immature zygotic embryos with the two 2,4-D concentrations assayed were identified with the letters A to D.

Stage	Length of embryo (mm)	Length of scutellum (mm) / Appearance	2,4-D (mg/l)	
			1 mg/l	2 mg/l
H	≅ 1.54	≅ 1.30 / Transparent	A	C
W	≅ 2.23	≅ 2.00 / Opaque	B	D

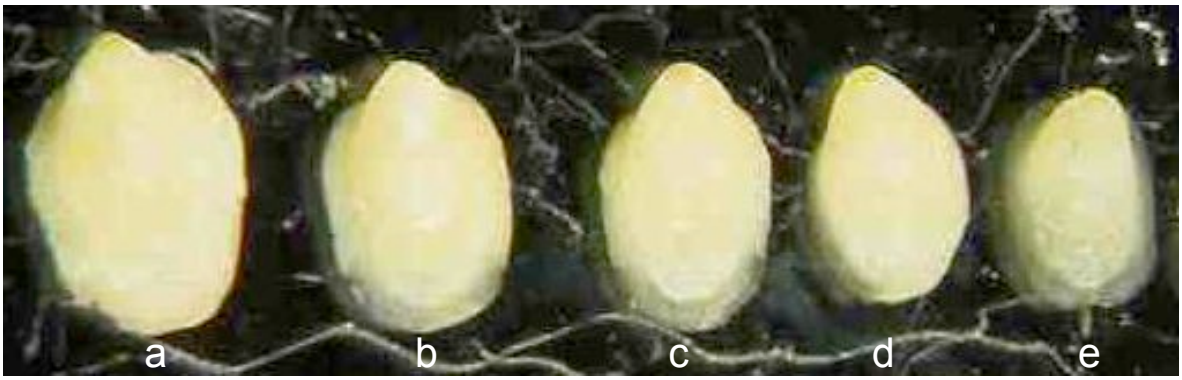

Figure 1. Five different developmental stages of immature zygotic embryos of wheat. Letters a and b represent stage W and letters c-e represent stage H, according to He *et al.* (1986).

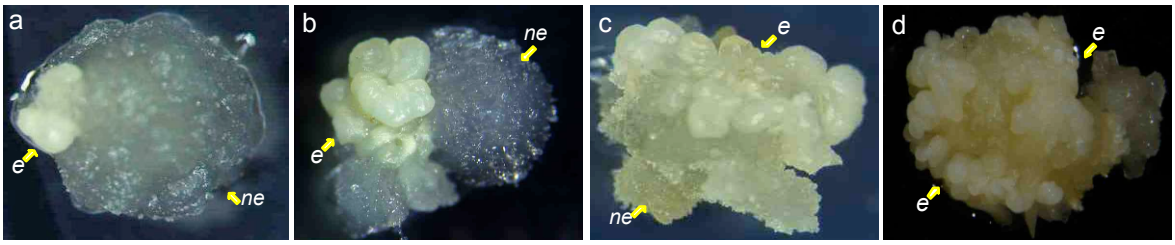

Figure 2. Scutellar callus three weeks after induction of immature embryos. Callus showing embryogenic (e) and nonembryogenic (ne) structures. Estimated percentage of callus proliferation on the scutellum surface: a) 20%; b) 45%; c) 70% and d) 100%.

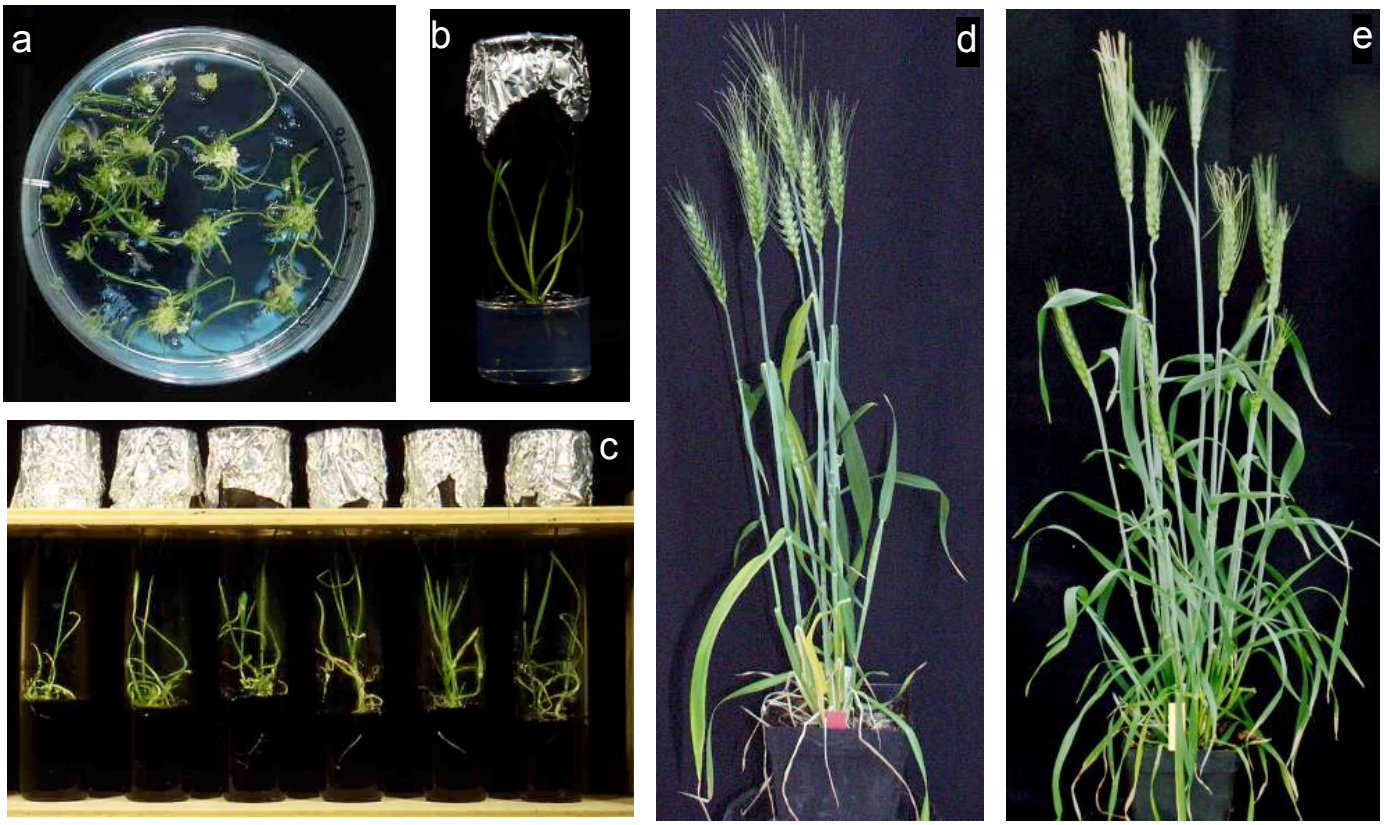

Figure 3. *In vitro* regeneration of wheat: (a) Regeneration on MSB-medium, (b) Continuation of the regeneration in small glass (c) Root formation in 190-2 medium. Greenhouse phase: (d) Mature plant derived from embryogenic scutellar callus and (e) seed-derived control plant of the cultivar *Klein Brujo.*

RESULTS

Induction of scutellar callus

A total of 10091 immature wheat embryos were used as starting material. Explants were able to produce both embryogenic and non-embryogenic calli. Embryogenic calli were nodular and solid, with a white to pale yellow surface, whereas non-embryogenic calli were soft, watery, and translucent (Fig. 2). After 3 weeks of culture on induction medium, all the spring wheat cultivars used, except *Prointa Elite,* produced embryogenic scutellar calli. Induction of scutellar embryogenic calli was significantly dependent on the culture conditions (Chi-square-test X^2 = 1017.5; p<0.001), as revealed by the rates of callus formation. The capacity of embryogenic callus formation varied widely among genotypes, ranging from 20.5 % to 85.7 % under culture condition A, from 0.0 % (five genotypes) to 46.8 % under culture condition B, from 10.1 % to 86.4 % under culture condition C, and from 0.0 % (two genotypes) to 89.3 % under culture condition D (Table 3). Under culture condition A, production of embryogenic calli was higher than 75 % with five genotypes (*Klein Brujo, Klein Pegaso, Klein Dragón, Prointa Puntual, Prointa Federal*), under culture condition C, with two of the genotypes (*Klein Dragón, Prointa Imperial*), and under condition D with one genotype (*Prointa Federal*). Under culture condition B, none of the genotypes reached 50% (Table 3).

Other factors influencing the formation of embryogenic calli from immature embryos were the stage of development and the concentration of 2,4-D. Immature donor embryos of developmental stage H and 1 mg 2,4-D/l medium (culture condition A) produced the best results, whereas embryos of the same stage and 2 mg 2,4-D (culture condition C) produced the second best results, being the difference between the two conditions statistically significant (Table 3). In contrast, increasing the concentration of 2,4-D from 1 to 2 mg/l medium had consistently positive effects when immature donor embryos of stage W were used (Table 3).

Capacity of callus proliferation of the cells of the scutellum surface

Callus proliferation of the cells of the scutellum surface was estimated and given in percent of the total scutellum surface (Fig. 2). The capacity of scutellum surface cells to form calli varied with the culture conditions and the genotype: from 30.0 % to 84.4 % under culture condition A, from 0.0 % to 63.3 % in culture condition B, from 25.0 % to 71.2 % in culture condition C, and from 25.0 % to 67.0 % in culture condition D (Table 3). The differences between the individual conditions were highly significant (Chi-square-test X^2 = 16.5; p<0.01). However, callus proliferation capacity varied less with the culture conditions than callus formation capacity as such (Table 3). The developmental stage of the immature embryos, however, was more crucial, being stage H significantly better than stage W (Table 3). On the other hand, the concentration of 2,4-D was less important for callus proliferation. Like the capacity of scutellar embryogenic callus formation, culture condition A produced the highest callus proliferation (two genotypes above 75%, *Klein Brujo, Klein Dragón*, and 11 above 50%), whereas culture condition C produced the second highest callus proliferation (11 genotypes above 50%, but none above 75%). A significant correlation between scutellar embryogenic callus formation and callus proliferation on the scutellum surface was observed under culture conditions A (r = 0.56; p = 0.006) and C (r = 0.47; p = 0.030).

Precocious germination of immature embryos

Precocious germination of the immature zygotic donor embryos varied among genotypes, ranging from 0.0 % to 32.1 % in culture condition A, from 0.0 % to 78.4 % in culture condition B, from 0.0 % to 31.4 % in culture condition C, and from 0.0 % to 97.7 % in culture condition D (Table 3). Even the otherwise not responding immature embryos of *Prointa Elite* showed precocious germination. Most failures of precocious germination were observed under the conditions C (11 out of 22 genotypes) and D (5 out of 22 genotypes) (Table 3). Both the culture conditions (Chi-square-test X^2 = 985.7; p<0.001) and the genotype influenced the extent of precocious germination (Table 3). Developmental stage H and 2 mg 2,4-D/l medium (culture condition C) was optimal for the inhibition of precocious germination. Differences with the other conditions were highly significant (Table 3). The frequency of precocious germination was highest in culture condition B.

Plant regeneration

The potential of scutellar embryogenic calli to regenerate whole plants after transfer to modified MSB medium (Fig. 3a and b) was investigated. Plant regeneration from scutellar embryogenic calli ranged from 46.4 % to 100 % under culture condition A, from 0.0 % to 100 % under culture condition B, from 30.0 % to 95.2 % in culture condition C, and from 0.0 % to 100 % in culture condition D (Table 4). Remarkably, under conditions A and C, all genotypes (but not all calli) produced plants. The composition of the callus induction medium and the developmental stage of the immature donor embryos influenced plant regeneration (Chi-square-test X^2 = 139.8; p<0.001). As expected, the genotype was also important. Culture condition A, i.e. immature embryos of stage H in a medium with 1 mg 2,4-D/l, was the best combination for the subsequent regeneration of plants (14 out of 21 = 66.6 %), which was triggered by transfer of the calli to regeneration medium. Developmental stage W in the same induction medium (culture condition B, 3 out of 21 = 14.3 %), as well as stage H cultivated in induction medium with 2 mg 2,4-D/l (culture condition C, 4 out of 21 = 19.0 %) were significantly less efficient (Table 4). For culture condition A, 20 genotypes showed regeneration efficiency over 50%, three of which (*Klein Brujo, Inta Huenpan,* and *Prointa B. Remodon*) reached 100%. For culture condition C, 19 genotypes showed regeneration efficiency over 50%, but none reached 100 % (Table 4). We found no relationship between scutellar embryogenic callus induction and plant regeneration capacity of the callus. We observed that genotypes exhibiting relatively poor callus induction frequency did not necessarily produce fewer regenerable calli (Table 3 and 4).

Table 3. Effect of the combination of the two developmental stages of immature zygotic embryos with the two 2,4-D concentrations (culture conditions A, B, C and D) on the frequency of scutellar callus induction, callus proliferation on the scutellum and precocious germination from twenty two Argentine wheat genotypes.

	Scutellar callus induction (formation) (%)[1, 3]				Callus proliferation on the scutellum (%)[4]				Precocious germination (%)[2,5]			
Cultivar	A	B	C	D	A	B	C	D	A	B	C	D
BCh.	62.0	46.8	48.3	35.7	39.0	34.0	48.0	33.3	0	0	0	31.0
BP.	59.7	0	43.6	9.4	55.0	0	49.0	45.0	6.3	66.0	0	5.7
KC.	20.5	0	10.1	8.6	31.0	0	38.9	35.0	23.8	27.5	0	6.9
KP.	84.7	19.1	48.8	10.7	50.0	34.1	50.6	30.0	3.4	27.0	0	23.2
KCa.	19.8	23.2	40.7	11.8	52.0	38.0	43.0	39.0	14.8	25.6	0	7.9
KDE.	38.5	32	18.2	26.4	52.0	41.1	55.0	32.1	4.8	9.6	0	15.1
KB.	84.8	33.6	72.5	35.9	77.0	40.4	71.2	48.2	0.6	36.7	2.5	25.0
KE.	31.4	0	45.5	2.8	30.0	0	25.0	25.0	23.5	72.1	9.1	86.1
KV.	68.3	8.8	63.3	15.4	52.1	33.3	63.3	33.3	8.1	34.1	0	7.7
KD.	85.7	31.3	76.1	29.4	84.4	58.9	67.9	49.1	5.9	29.0	0	29.4
KEs.	64.6	22.1	61.5	1.8	53.9	45.6	63.5	37.5	9.5	37.1	5.1	43.0
PO.	27.7	7.1	58	4.7	59.6	31.3	56.0	37.5	4.2	48.2	5.8	48.8
PE.	0	0	0	0	0	0	0	0	32.1	37.2	31.4	23.3
DEI.	57.1	13.3	55.6	53.7	63.3	52.9	69.3	54.5	21.4	65.6	0	0
PI.	53.2	0	86.4	0	38.0	0	39.5	0	19.2	50.0	0	0
PA.	33.8	28.6	29.6	0	42.9	62.5	43.8	0	21.5	0	11.1	0
PG.	58.7	16.7	66.7	53.2	55.6	63.3	57.2	67.0	7.7	14.0	4.4	0
PF.	78.3	5.0	58.6	89.3	46.9	37.5	56.5	37.9	2.4	67.5	0	2.9
IH.	32.6	11.1	13.2	10	50.0	43.7	35.7	31.3	2.3	29.2	9.4	0
PP.	80.4	14.9	72.6	1.2	65.4	40.9	50.9	50.0	23.9	78.4	26.8	97.7
PBR.	46.2	2.9	64.5	25	40.0	33.3	47.7	43.8	8.8	28.4	12.9	37.5
PC.	31.5	0	30.8	10.5	30.0	0	39.6	25.0	6.9	67.5	12.8	36.8
Means ± SD	51.0 ± 24.4^{ae}	14.4 ± 13.7^{bg}	48.4 ± 23.2^{cf}	19.8 ± 22.6^{dh}	48.6 ± 17.7^{ae}	31.4 ± 21.6^{bf}	48.7 ± 16.0^{ce}	34.3 ± 17.1^{df}	11.4 ± 9.4^{ae}	38.7 ± 23.1^{bg}	6.0 ± 8.8^{cf}	24.0 ± 26.9^{dh}

[1] Number of immature embryos forming embryogenic callus / number of immature embryos X 100

[2] Number of immature embryos with germinating zygotic embryo / number of immature embryos X 100

[3 a-h] Means followed by the same letter are not significantly different at 1 % level.

[4 a-f] Means followed by the same letter are not significantly different at 5 % level

[5 a-h] Means followed by the same letter are not significantly different at 0.1 % level

Table 4. Frequency of plant regeneration capacity, culture efficiency and number of plants regenerated per embryo (precultured under condition A, B, C and D) cultured on MSB-medium.

	Plant regeneration capacity (%)[1, 4]				Number of plants regenerated[2, 5]				Culture efficiency (%)[3, 6]			
Cultivar	A	B	C	D	A	B	C	D	A	B	C	D
BCh.	93.3	73.6	93.7	74.2	2.1	1.6	2.7	1.7	57.8	34.4	45.3	26.5
BP.	77.7	-	57.9	55.5	7.9	-	5.6	4.4	46.4	-	25.2	5.2
KC.	61.3	-	57.6	48	5.3	-	5.3	2.9	12.6	-	5.8	4.1
KP.	72.5	60.0	64.7	72.5	2.9	2.6	5.0	1.8	61.4	11.5	31.6	7.8
KCa.	46.4	0	68	12.5	1.8	0	2.0	2.0	9.2	0	27.7	1.5
KDE.	73.3	66.6	68	0	6.1	6	6.4	0	28.2	21.3	12.4	0
KB.	100	100	76.3	100	9.5	7.6	5.6	5.0	84.8	33.6	55.3	35.9
KE.	78.8	-	65	50	7.5	-	6.6	4.6	24.7	-	29.6	1.4
KV.	96.3	100	95.2	100	6.8	4.2	8.3	6.4	65.8	8.8	60.3	15.4
KD.	97.0	74.2	30	42.9	9.5	4.5	1.7	4.8	83.1	23.2	22.8	12.6
KEs.	54.3	8.6	37.9	25.8	5.7	3.3	4.9	4.8	35.1	1.9	23.3	0.5
PO.	94.4	100	72.7	56.3	3.8	3.3	3.4	3.3	26.2	7.1	42.2	2.6
PE.	-	-	-	-	-	-	-	-	-	-	-	-
DEI.	92.3	81.2	91.7	87.1	5.8	2.0	1.6	1.8	52.7	10.8	51.0	46.8
PI.	88.6	-	64	-	3.7	-	3.4	-	47.1		55.3	
PA.	85.7	56.3	88.8	-	2.3	2.0	2.3	-	29.0	16.1	26.3	
PG.	65.7	56.2	66.5	52.9	2.5	1.8	3.6	3.1	38.6	9.4	44.4	28.1
PF.	93.1	0	83.3	66.6	5.6	0	2.3	3.2	72.9	0	48.8	59.5
IH.	100	65.5	75	57.1	2.3	3.1	2.0	2.2	32.6	7.3	9.9	5.7
PP.	85.5	95.2	83.9	80.6	8.0	7.4	7.6	6.0	68.7	14.2	60.9	1.0
PBR.	100	82.3	93.5	80	2.9	6.0	2.1	2.4	46.2	2.4	60.3	20.0
PC.	92.3	-	79.1	66.6	4.2	-	3.4	2.4	29.1		24.4	7.0
Means ± SD	83.3 ± 15.8^{ae}	63.7 ± 33.8^{bf}	72.0 ± 17.4^{ce}	59.4 ± 26.7^{df}	5.1 ± 2.5^{ab}	3.5 ± 2.3^{ac}	4.1 ± 2.0^{ab}	3.3 ± 1.7^{ac}	45.3 ± 21.8^{ae}	12.6 ± 10.8^{bf}	36.3 ± 17.4^{ce}	14.8 ± 17.2^{dg}

[1] Number of regenerable calli / number of calli induced X 100.
[2] Number of regenerants per embryo cultured gave as mean numbers
[3] Number of regenerable calli / number of embryos cultured X 100 (regenerable callus = nodular callus with green spots).
[4 a-f] Means followed by the same letter are not significantly different at 0.1 % level
[5 a-c] Means followed by the same letter are not significantly different at 5 % level
[6 a-g] Means followed by the same letter are not significantly different at 1 % level

Number of plants arising from scutellar embryogenic callus

Because usually more than one embryogenic callus was formed on the surface of the scutellum, differing numbers of plants were expected from one immature embryo (Fig. 3a). Effects of the genotype as well as of the culture conditions during scutellar callus induction were investigated. Culture condition A (1.8 – 9.5, average 5.1 plants per donor embryo) again proved better than the other three conditions, which gave rather similar results (B: 1.6 – 7.6, average 3.5; C: 1.6 – 7.6, average 4.1; D: 1.7 – 6.4, average 3.3) (Table 4). However, the differences were not statistically significant (Kruskal-Wallis-test: $X^2 = 57.4$ $p = 7.3$). The number of plants regenerated and the plant regeneration capacity of scutellar embryogenic calli were not significantly correlated.

Because of the high number of plants, plant samples were taken randomly from each cultivar and culture condition. Each plant regenerated was transferred into soil in greenhouse conditions (*ex vitro*) after *in vitro* root promotion (Fig. 3c). In that phase, neither the cultivar nor the culture condition influenced the morphogenetic development (Fig. 3d). All plants matured normally and set fertile seeds after approximately three months.

Overall efficiency of the four culture conditions

Given the wide range of embryogenic callus formation displayed by the 22 cultivars investigated and their differing capabilities for plant regeneration, the significant differences in overall efficiencies in plant regeneration were not unexpected (Chi-square-test $X^2 = 31.9$; $p<0.001$). Comparison of the influence of culture conditions A – D showed that younger immature donor embryos (stage H) were better than more advanced ones (stage W), irrespective of the concentration of 2,4-D in the medium (Table 4). With donor embryos of stage H, the lower concentration of 2,4-D (1 mg 2,4-D/l) was better than the higher one (2 mg 2,4-D/l), whereas with donor embryos of stage W, the higher concentration was slightly more effective. The influence of the genotype was overridden by the culture conditions, i.e. the values of overall efficiency displayed by the 22 cultivars were not identical after the four culture conditions, not even when the same developmental stage H or W was considered: while *Klein Brujo* (84.8 %), *Klein Dragón* (83.1 %) and *Prointa Federal* (72.9 %) were the best after preculture under condition A, *Prointa Puntal* (60.9 %), *Prointa B. Remodon* (60.3 %) and *Klein Volcán* (60.3 %) were the best after preculture under condition C. When using developmental stage W, the three best varieties were *Prointa Federal* (59.5 %), *Don Ernesto Inta* (46.8 %) and *Klein Brujo* (35.9 %) after preculture under condition D, and *Buck Charrua* (34.4 %), *Klein Brujo* (33.6 %) and *Klein Dragón* (23.2 %) after preculture under condition B.

In decreasing order, the four cultivars with highest scutellar embryogenic callus were *Prointa Federal* (89.3 %) under culture condition D, *Prointa Imperial* (86.4 %) under culture condition C, and *Klein Dragón* (85.7 %) and *Klein Brujo* (84.8 %) under culture condition A. Nevertheless, this order changed when the culture efficiency and the number of plants regenerated were considered. The first place was occupied by *Klein Brujo* with 84.8 % and 9.5 respectively, followed by *Klein Dragón* (83.1 %, 9.5), *Prointa Federal* (59.5 %, 3.2) and *Prointa Imperial* (55.3 %, 3.4).

We may conclude that among the 22 spring wheat varieties investigated, *Klein Brujo* was the best when pre-cultured under condition A, but also yielded moderate to good results under the other three conditions. *Klein Dragón* was the second best, with similar good results under condition A but not under the other three conditions.

DISCUSSION

Variables and their interactions in callus formation and plant regeneration

The data presented show that induction of scutellar callus, precocious germination of immature embryos, capacity of scutellar embryogenic callus to grow into plants and the entire culture efficiency, all depended on the genotype, the stage of development of the immature embryo, the concentration of 2,4-D in the medium, and the interaction(s) between them. While the capacity of scutellar calli to develop new shoots under the experimental conditions applied depended only on the genotype, callus proliferation on the scutellum also depended on the developmental stage of the immature embryos.

The significant influence of the genotype on both processes has also been observed in other studies (Carman *et al.*, 1987; Fennell *et al.,* 1996; Özgen *et al.,* 1998; Viertel *et al.,* 1998; Barro *et al.,* 1999; Dağüstü, 2008). In this work, the importance of the developmental stage was given special attention in combination with the concentration of the hormone in the medium. In agreement with that reported by León *et al.* (2006), the younger stage of the immature embryo was more effective and the effect of maturation of the embryo could not be counteracted by

doubling the 2,4-D concentration of the medium. The higher 2,4-D concentration was more effective in the promotion of callus formation only if older embryos (stage W) were used, a finding which is line with that reported by Almouslem *et al.* (2005). Using young immature embryos (stage H), the higher concentration of the auxin in the medium usually inhibited both morphogenetic processes, as well as the precocious germination of the embryo. This finding was conflicting because callus formation and propagation and regeneration of plants from the calli were more effective with the lower concentration of auxin in the medium, which also promoted precocious germination. Either a slight or no inhibitory effect of a higher 2,4-D concentration on embryogenic callus formation and plant regeneration was reported by Viertel *et al.* (1998) for 18 German spring wheat varieties. In agreement with our results, Barro *et al.* (1999) found that the concentration of 2,4-D played a crucial role and that the lower concentration was better for scutellar embryogenic callus formation and regeneration. A loss of responsiveness to the hormone with the advance of maturation of the embryo cannot be ruled out as an explanation of the phenomena observed. Inhibition of the increase in precocious germination as an indicator of the advancing maturation required higher auxin concentration in the medium.

Is there a correlation between the potentials of callus induction and plant regeneration from the calli?

Vegetative propagation of wheat varieties by tissue culture methods depends on both a high potential for embryogenic callus production and a high capacity of plant regeneration from the calli. In the Argentine spring wheat cultivars studied, the regeneration rate of plants from calli was high, but the scutellar callus formation rate of the immature embryos was rather low. Not the entire surface of the wheat-scutellum is capable of forming embryogenic callus (Scott *et al.,* 1990). In that respect, callus proliferation on the scutellum surface is an important parameter for biolistic transformation of wheat. However, only Viertel *et al.* (1998) studied this issue. These authors found an increase in the portion of the callus producing scutellar surface when 1 mg/l instead of 2 mg/l of 2,4-D was used in the medium. Like in our present work, Viertel *et al.* (1998) also reported that there was a positive correlation among the frequency of immature embryos forming embryogenic callus and the callus proliferation on the scutellum surface. In the present study, with a substantial collection of Argentine spring wheat cultivars, the young developmental stage of the immature embryo produced a higher percentage of callus proliferation, although the variation between the genotypes was considerable.

Even with the best of our protocols (culture condition A), the overall efficiency of the entire tissue culture approach did not exceed the values reported for South American wheat cultivars (Chowdhury *et al.,* 1991).

In wheat, like in other plants, the variation in tissue culture response is assumed to be controlled by the interaction of a large number of genes (polygenes) (Fennell *et al.,* 1996; Varshney and Altpeter, 2001). With the 22 Argentine spring wheat varieties investigated in this work, we found no relationship between embryogenic callus formation and regeneration capacity. This is in agreement with observations by Sears and Deckard (1982), Chowdhury *et al.* (1991) and Özgen *et al.* (1996, 1998). It appears that both phenomena are controlled by different genes or gene combinations. However, Viertel *et al.* (1998), Barro *et al.* (1999) and Dağüstü (2008) reported a close correlation between both processes and, at the current state of knowledge, there is no explanation for this contradiction.

Reports about the numbers of plants regenerated per cultured explant differ significantly, a fact that may be explained by the genotypic variation and the culture conditions. In the Argentine spring wheat cultivars studied, the influence of the culture conditions appeared to be restricted to the early stages of the tissue culture protocol. Once shoot regeneration from calli was induced, neither the developmental stage of the immature embryo nor the auxin concentration in the medium played a particular role in the number of plants regenerated.

The Argentine wheat genotypes analyzed regenerated a low number of plants per embryo (1.6 to 9.5) in comparison with other reports (Fennell *et al.,* 1996: 6 to 42; Viertel *et al.,* 1998: 1 to 19; León *et al.,* 2006: 2.3 to 16.8). Only the genotypes examined by Varshney and Altpeter (2001) showed results (1.01 to 9.2) similar to those of the present study.

The present study aimed to gain knowledge on the best conditions for successful genetic transformation of spring wheat. Embryogenic scutellar calli are the most widely used target tissue for gene transfer in wheat. As the medium is important for embryogenesis in these calli, it could also be used as selection medium for transgenic plants. Among the 22 Argentine spring wheat genotypes studied, *Klein Brujo*

and *Klein Dragón* seemed to be the most promising. The present study also showed that standard culture conditions cannot be applied for all genotypes. This result concurs with the hypothesis of Redway *et al.* (1990), which establishes that the differences in genotype response are physiological in nature and thus require adjustment of the *in vitro* culture conditions and media.

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OXIDATIVE STRESS AND COMET ASSAY IN TISSUES OF MICE ADMINISTERED GLYPHOSATE AND AMPA IN DRINKING WATER FOR 14 DAYS

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ABSTRACT

Excessive amounts of the herbicide glyphosate are incorporated daily to the soil and the ecosystems. AMPA is its major environmental breakdown product. In this study we determined the levels of thiobarbituric acid reactive substances (TBARs); quantified superoxide dismutase (SOD) and catalase (CAT) activity in liver, kidney, lung and heart, and performed the comet assay in blood and liver of mice administered glyphosate (40 or 400 mg/kg/day) or AMPA (100 mg/kg/day) in drinking water for 14 days. Exposure to glyphosate 400 mg/kg induced a statistically significant ($p < 0.05$) decrease of SOD activity in heart and an increase in CAT activity in kidney. In the comet assay there were statistically significant differences in all the treatments and tissues studied in comparison to control animals ($p \leq 0.01$). The major results of this study were that mice administered glyphosate or AMPA in drinking water for 14 days induced a significant increase in DNA damage in liver and blood but minor effects on oxidative stress parameters. DNA effects on liver and blood indicate that these compounds could be of concern in terms of their potential to damage the genetic material, and that oxidative stress does not seem to be the mechanism causing that effect.

Key words: pesticides, mice, genotoxicity

RESUMEN

Cantidades excesivas del herbicida glifosato son incorporadas diariamente a los suelos y a los ecosistemas. Su principal metabolito ambiental es el ácido amino-metil-fosfónico (AMPA). En el presente estudio se determinaron los niveles de sustancias reactivas al ácido tiobarbitúrico (TBARs), se cuantificaron las actividades de superóxido dismutasa (SOD) y catalasa (CAT) en hígado, riñón, pulmón y corazón, y se realizó el ensayo cometa en sangre e hígado de ratones expuestos a glifosato (40 o 400 mg/kg/día) o AMPA (100 mg/kg/día) durante 14 días en el agua de bebida. La exposición a glifosato 400 mg/Kg indujo un descenso estadísticamente significativo ($p < 0,05$) en la actividad SOD en corazón y un aumento de la actividad CAT en riñón. En el ensayo cometa se observaron diferencias estadísticamente significativas en todos los tratamientos y tejidos estudiados en comparación con los animales controles ($p \leq 0,01$). Los resultados principales de este estudio son que los ratones expuestos a glifosato o AMPA en el agua de bebida durante 14 días presentaron un incremento significativo en los niveles de daño al ADN en hígado y sangre, y efectos menores en los parámetros de estrés oxidativo. Los efectos sobre el ADN indican que estos compuestos podrían ser de preocupación en términos de su potencialidad para dañar el material genético, y que el estrés oxidativo no parece ser el mecanismo que conduce a ese daño.

Palabras clave: pesticidas, ratones, genotoxicidad

INTRODUCCIÓN

Glyphosate is the active ingredient of a broad spectrum, non-selective systemic herbicide, used in post-emergence treatments to control annual and/or perennials weeds in agriculture, forestry and landscape environments (Malik *et al.*, 1989; Williams *et al.,* 2000). Regulatory agencies have carried out reviews on the safety of glyphosate used in its different commercial formulations and they had concluded that there is no indication of human health concern (EPA, 1993; WHO, 1994). However, other independent and recent reports indicate that both the herbicide and its metabolite AMPA would not be as safe as originally stated (Iserning, 2004; Çağlar and Kolankaya, 2008). It has been shown that glyphosate is able to induce genotoxic (Li and Long, 1988; Rank *et al.,* 1993; Van de Waart *et al.*, 1995; Bolognesi *et al.,* 1997; Kier *et al.,* 1997; Lioi *et al.,* 1998a; Monroy *et al.,* 2005) and/or oxidative damage (Lioi *et al.,* 1998b.; Pieniazek, *et al.*, 2004; Beuret *et al.,* 2005; Gehin *et al.,* 2005) *in vitro* and *in vivo.* Genotoxic evaluations include increase in micronuclei, sister chromatid exchanges and DNA damage (Vigfusson *et al.,* 1980; Bolognesi *et al.,*1997; Clements *et al.,* 1997; Lioi *et al.,* 1998a, 1998b; Šiviková and Dianovský, 2005; Mañas *et al.,* 2006; Poletta *et al.,* 2008; Mañas *et al.,* 2009b).

Aerobic and anaerobic environmental degradation of the parental molecule originates at least six metabolites, being the amino methyl phosphonic acid (AMPA), the major one (Forlani *et al.*, 1999). The AMPA and glyphosate half-life in the soil ranges from 76 to 240 days and from 2 to 97 days, respectively (Battaglin *et al.*, 2005). In addition to the greater soil persistence of AMPA when compared with that of glyphosate, there is evidence indicating that AMPA can cause groundwater contamination (Botta *et al.*, 2009). A scarce number of studies points out to the genotoxic effects of AMPA (Mañas *et al.*, 2009a). Moreover, in very few of these studies the sub-chronic oral exposure to glyphosate have been evaluated (Benedetti *et al.,* 2004; Dallegrave *et al.,* 2007; Çağlar and Kolankaya, 2008).

The overproduction of reactive oxygen species (ROS) can be implicated in DNA damage. As a result of the ROS attack to the DNA, permanent changes in the genetic material are the first step in the processes of mutagenesis, carcinogenesis and aging. ROS can cause single and double strand DNA breaks, crossovers and changes in purines, pyrimidines, or deoxyribose (Beuret *et al.*, 2005; Astiz *et al.*, 2009). Also, it was informed that pesticides can induce oxidative stress through the generation of ROS and/or alteration in antioxidant enzyme systems. Therefore, ROS could be involved in the toxicity of glyphosate-based pesticides (Gehin *et al.*, 2005).

Because of the discrepancies and even opposite results reported in the literature regarding the potential risks to human and animal health and to the environment posed by the use of glyphosate-based herbicides, it is important to evaluate the effects of glyphosate and AMPA as potential inducers of oxidative stress and genotoxic damage in mammalian tissues. In the present study we evaluated oxidative stress and genotoxicity of glyphosate and AMPA in mice, administered in drinking water for 14 days. To accomplish this goal we determined Thiobarbituric acid reactive substances (TBARs) and quantified superoxide dismutase (SOD) and catalase (CAT) activity in liver, kidney, heart and lung. The evaluation of the genotoxic potential of glyphosate and AMPA was performed in the same animals by the comet assay in peripheral blood and liver.

MATERIALS AND METHODS

Chemicals

Analytical grade glyphosate [N-(phosphonomethyl) glycine], CAS 1071-83-6 (96%), and analytical grade AMPA, CAS 1066-51-9 (99%) were purchased from Sigma-Aldrich, Argentina. Normal melting point agarose (NMP) and low melting point agarose (LMP) were acquired from Promega, Argentina. All other chemicals and solvents used in this study were of analytical grade.

Animals

We used 24 Balb C mice approximately 45 days of age from the animal facility of the Faculty of Agronomy and Veterinary Medicine, National University of Río Cuarto. Four groups of six animals each (a control group, two groups treated with glyphosate and a group exposed to AMPA) were used for TBARs, SOD and CAT determinations and the comet assay. They were housed in pairs in 12 plastic cages with food and water *ad libitum*. During a period of acclimation of seven days prior to the beginning of the experience, we measured the volume of water ingested per day, every two animals per cage, in order to determine the concentration of the solutions of glyphosate and AMPA to

be supplied to each group. Mice exposed to the herbicide and its metabolite received approximately 40 or 400 mg/kg/day of glyphosate (G40 and G400 groups) and 100 mg/kg/day of AMPA (A100 group), both compounds were provided orally (p.o.) via drinking water. The doses selected were similar to those employed by other authors in toxicology (including genotoxicology) and toxicokinetics studies (Rank *et al.*, 1993; Bolognesi *et al.*, 1997; Benedetti *et al.*, 2004; Dallegrave et al., 2007; Çağlar and Kolankaya, 2008; Mañas *et al.*, 2009a and 2009b; Anadon *et al.*, 2009).

The solutions were freshly prepared every two days and the volume of water ingested per day was measured through the trial. Glyphosate and AMPA were dissolved in sterile fresh water, and pH was adjusted to 7.2-7.4 with 0.1 M NaOH. The control group received only the aqueous vehicle. After a period of 14 days from the beginning of the experiment, peripheral blood was drawn from the tail vein to perform the comet assay. Subsequently the animals were euthanized by cervical dislocation; the dissection and removal of organs (heart, lungs, liver, and kidneys) was carried out for oxidative stress determinations (TBARs, SOD and CAT). These tissues were frozen immediately at -80 °C until analysis. Part of the liver tissue was immediately homogenized with phosphate buffer (pH 7.4) to perform the comet assay. All determinations were performed by triplicate.

Single cell gel electrophoresis assay in mice blood and liver

The protocol followed the general guidelines proposed by Singh *et al.* (1988), with minor modifications. The slides were fixed in absolute ethanol, stained with ethidium bromide, and scored using fluorescence microscopy. Images of 100 "nucleoids" counted for each animal were captured with a camera attached to the fluorescence microscope and linked to the Comet Score 1.5 software. Tail moment (TM), percentage of DNA in tail (% of DNA) and tail length (TL) were used to estimate DNA damage (arbitrary units).

TBARs, SOD and CAT determinations

Tissue homogenates (10%) were prepared in chilled 0.05M potassium phosphate buffer, pH 7.4. TBARs concentrations, expressed as nmol of malondialdehyde (MDA)/g of tissue, were measured by a spectrophotometer at 532 nm in liver and kidney homogenates using the method of Marcinčák et al. (2003). TBARs concentrations were determined using a standard curve at different concentrations of MDA versus optical density, individually prepared for each tissue. Superoxide dismutase activity was assessed spectrophotometrically in the supernatant of liver homogenates according to the method of Misra and Fridovich (1972). One unit of enzymatic activity has been defined as the amount of enzyme capable of causing 50% inhibition of auto-oxidation of epinephrine. Catalase activity was measured at 240 nm by the decomposition of the H_2O_2 as described by Glick (1954).

Statistical analysis

Statistical analysis was performed using Prism software (PRISM, 1997). The Kolmogorov-Smirnov test was performed to verify if the results follow a normal distribution. ANOVA followed by Dunnet test, or Kruskal-Wallis followed by Dunns test were performed for data with and without normal distribution respectively.

RESULTS

The volume of water ingested per animal/day did not show statistically significant differences (ANOVA, $p>0.05$) among the experimental groups throughout the experiment (14 days) (data not shown). The results of the TBARs, SOD and CAT determinations in tissues of mice after 14 days of drinking water with glyphosate 40 and 400 mg/kg/day and AMPA 100 mg/kg/day are shown in Figure 1. Concentrations of MDA/ g of tissue from the animals of the control group were 4.57 ± 3.06 nmols/ g liver, 17.57 ± 4.20 nmols/ g kidney, 5.04 ± 3.60 nmols/ g lung and 18.34 ± 11.40 nmols/ g heart. There were no statistically significant differences in the concentrations of MDA/ g of tissue among the control, G40, G400 and A100 groups. SOD activity in the animals of the control group was 173.40 ± 18.32 units/ g liver, 400.20 ± 51.00 units/ g kidney, 235.60 ± 69.35 units/ g lung and 213.00 ± 50.70 units/ g heart. There were statistically significant differences in SOD activity in heart in the G400 group ($p <0.05$). SOD activity was lower in heart in the G400 group (102.50 ± 46.42), compared with that in the other treatments ($p <0.05$). CAT activity in the animals of the control group was 34.37 ± 7.04 nmols/ g liver, 13.48 ± 4.89 nmols/ g kidney, 73.40 ± 30.28 nmols/ g lung and 28.69 ± 10.13 nmols/ g heart. There were statistically significant differences in CAT activity in kidney in the G400 group (33.21 ± 14.83). The results for the comet

assay in peripheral blood and liver of Balb C mice are shown in Table 1. Statistically significant differences in tail intensity, length and moment were found between each treatment and the control group ($p<0.0001$, Dunn's test), except in liver tissue in the G40 treatment ($p<0.05$ and $p<0.001$ in tail moment and tail length parameters).

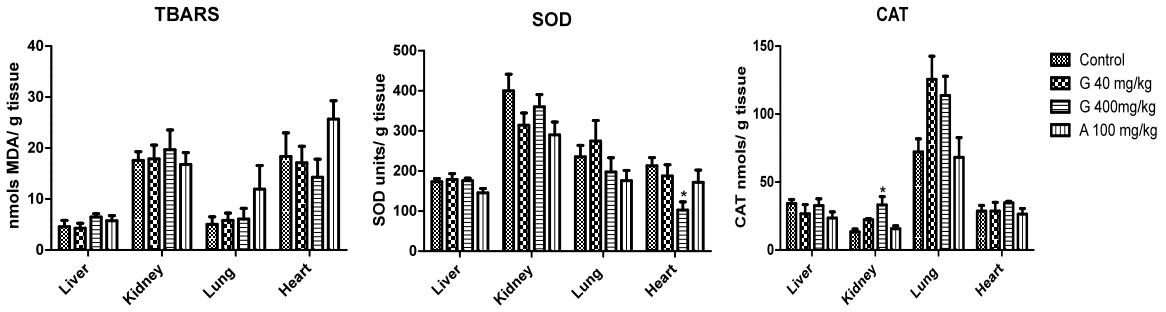

Figure 1. Changes in the levels of TBARs, SOD and CAT activity in liver, kidney, lung and heart in mice administered Glyphosate or AMPA in drinking water during 14 days. n= 6 animals per group. $p<0.01$ respect to the control, Dunn test. G: glyphosate, A: AMPA. Each determination was performed by triplicate.

Table 1. Comet assay in blood and liver of mice administered Glyphosate or AMPA in drinking water during 14 days. n= 6 animals per group. *p< 0.05, **p< 0.001, ***p< 0.0001 respect to the control, Dunn test.

	Tail intensity mean ± SEM (arbitrary units)		Tail length mean ± SEM (arbitrary units)		Tail moment mean ± SEM (arbitrary units)	
	blood	liver	blood	liver	blood	liver
Control	± 1.73 0.85	± 1.04 0.82	± 7.02 6.48	± 2.91 1.09	± 2.98 1.08	± 7.14 3.41
14 days Glyphosate 40 mg/Kg	± 3.39*** 1.55	± 1.21 0.91	16.60*** ± 0.99	± 3.97** 3.25	± 8.54*** 7.82	± 7.92* 3.99
14 days Glyphosate 400 mg/Kg	± 3.64*** 1,17	± 1.62*** 0.71	16.68*** ± 0.69	± 9.36*** 4.92	± 9.06*** 5.15	20.59*** ± 15.47
14 days AMPA 100 mg/Kg	± 2.86*** 1,36	± 1.28*** 0.53	14.70*** ± 0.95	± 7.02*** 4.03	± 8.45*** 6.43	14.99*** ± 9.09

DISCUSSION

The presence of glyphosate or AMPA in drinking water did not affect its consumption in the volume of water ingested as no statistically significant differences were determined among the groups throughout the 14 days of the trial.

In the studied doses of the herbicide and its metabolite and in the four tested organs (liver, kidney, lung and heart), statistically significant differences in oxidative activity were only observed in G400 treatment. In animals that ingested glyphosate at a concentration of 400 mg/kg in drinking water for 14 days, SOD activity (units/ g tissue) decreased in kidney, lung and heart, although it was only statistically significant in heart (102. 50 ± 46.42⋆ SOD units/ g tissue). On the other hand, CAT activity increased in the same organs but it was statistically significant only in kidney (33.21 ± 14.83 ⋆ CAT nmols/ g tissue).

A decreased in SOD activity with an interesting biological significance was also observed in all organs of animals administered with AMPA100 mg/kg in drinking water, but without statistically differences; CAT activity showed no variation in this treatment. As it was presented, the activity in CAT enzyme significantly increased in the G400 group, but did not change in the G40 group, except for an increase in lung. Liver was the only organ that did not present any type of variations, neither in TBA reactive substances nor in SOD and CAT activity This suggests a better oxidative balance in this organ in a subchronic design since earlier studies on oxidative stress have shown that the levels of CAT increased in liver of mice treated with glyphosate intraperitoneally (ip) at a dose of 400 mg/kg (Mañas *et al.*, 2009b) within 1 h after a single ip administration. However, at 14 days oral exposure, liver CAT activity was recovered. These differences, after ip or oral exposure, in the enzymatic activity could be related to the toxicokinetic of glyphosate (mainly, the route of administration), as well as to the different experimental designs in terms of exposure time. Like any other chemical compound, glyphosate has a particular kinetic depending on the chosen route of administration. Intraperitoneal administration has a rapid and almost complete absorption, and provides a high bioavailability of the administered compound, whereas in oral administration the bioavailability is lower probably because the gastrointestinal absorption rate mostly depends on the physical and chemical characteristics of the administered compound. According to previously reviewed toxicokinetic studies (Williams *et al.*, 2000) glyphosate has a poor gastrointestinal absorption. The oral bioavailability, according to various authors, varies between 19% to 36% (Anadón et al., 2009). We may assume that the ip administration of 400 mg/kg of glyphosate resulted in a relatively high plasma concentrations of the herbicide in a short period of time, followed by the generation of ROS, which led to an increase in the hepatic activity of SOD and catalase. It is also possible that when glyphosate and AMPA were orally administered, a compensatory increase in SOD and CAT activity occurred due to ROS increase, followed by a decline in the levels of SOD and CAT until normalized levels over the course of days, and even fell below the control group levels in those animals exposed to 100 mg/kg of AMPA. Langiano and Martinez (2008) and Modesto and Martinez (2010) also reported temporary alterations in SOD and CAT hepatic activity in fish, with a compensatory effect of ROS levels by enzymatic activity. This was clearly observed in CAT and SOD determinations in our study in mice liver. The activity of both enzymes was higher in the previously ip administration design (Mañas *et al.*, 2009b) compared to the current design of oral administration of glyphosate at a dose of 400 mg/kg (Figure 1). This suggests that enzymatic activity could be normalized once ROS are removed; therefore the imbalance (oxidative stress) between ROS and antioxidant systems is corrected. Other authors have reported an increased lipid peroxidation in liver tissue of pregnant rats and their fetuses exposed to glyphosate, which demonstrates the potential of this herbicide to cause oxidative stress *in vivo* (Beuret *et al.*, 2004). To our knowledge, there are no published results related to the evaluation of AMPA as a possible inducer of oxidative stress, and no biological difference was detected in the assayed dose.

The single cell gel electrophoresis assay (comet assay) is a sensitive technique used to measure DNA damage in individual cells, and has been used both *in vitro* and *in vivo* to identify potentially genotoxic compounds of environmental use (Sasaki *et al.*, 2000; Moller, 2006). In the genotoxicity results obtained in the comet assay in blood in the present study, significant differences were determined between the three treatments and the control group in tail intensity, length and moment. Regarding the similar statistical significance in these three parameters evaluated

($p < 0.0001$) in almost all the groups, the higher absolute values were found in the G400 treatment.

It can be observed that tail moment values in the comet assay were similar for both glyphosate and AMPA treatments. This allows us to affirm that the scientific community concerned about the genotoxic effects of environmental xenobiotics should be worried about AMPA as much as glyphosate. Using the comet assay and an also *in vivo* design, Bolognesi *et al.* (1997) reported DNA damage in liver and kidney of mice exposed to glyphosate at 300 mg/kg by ip route. In a previous work in Balb C mice and after 1 hour of ip exposure to glyphosate at 400 mg/kg, we found a statistically significant increase in the percentage of genetically damaged blood cells (98.0 ± 1.1) (Mañas *et al.*, 2006). The top or maximum tolerated dose recommended for *in vivo* genotoxicity assays is the dose such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality. In multiple administration studies, a limit dose of 1000 mg/kg is used for 14 days or longer studies; if this dose is tolerated, it is recommended (ICH, 2012). This also means that recommended doses for genotoxicity testing are not related or calculated on the basis of environmental concentrations, not even for human or animal exposure. On the other hand, these are very scarce data in the literature regrading this point. In balb C mice we have previously observed lethality in preliminary studies using doses above the employed in the present work.

Some authors have discussed the role of the oxidative stress as a mechanism of action of some pesticides (Astiz *et al.*, 2009). Antioxidant defense mechanisms, including SOD and CAT, have a considerable importance because they are involved in protection against free radicals produced during oxidative stress. In this study, we have detected genotoxic changes in G40, G400 and A100 treatments; however, significant changes in the levels of SOD and CAT especially in heart and kidney were only caused by G400 exposure. This let us to suppose that the genotoxic effects of glyphosate and AMPA are much more important than the indicators of oxidative stress, therefore the latter does not seem to be related to the genotoxicity observed.

To our concern, the more relevant results of this study are those that demonstrate alterations that suggest genotoxicity in mice exposed to AMPA. Moreover, previously we have reported that AMPA induced genotoxic effects in all the assays performed: chromosomal aberrations, comet assay in Hep-2 cells and micronucleus test in mice (Mañas *et al.*, 2009b). Results from the present study provide the bases for future research using a broader number of doses of AMPA to assess oxidative stress and genotoxicity. Despite the fact that little is known about AMPA biological activity, regulatory agencies have determined that it is not of toxicological concern and, therefore, it is not included in risk assessments (EPA, 1993, WHO, 1994). However, our results from genotoxicity assays indicate that AMPA is a metabolite of risk from the toxicological point of view. AMPA residues, as well as glyphosate, are found as contaminants in the environment (Botta *et al.*, 2009, Hanke *et al.*, 2010; Schriks *et al.*, 2010). In this work we explored oral administration because is one of the most expected routes to glyphosate and AMPA exposure, besides the respiratory and dermal. We affirm that more sub-acute and sub-chronic studies need to be performed.

Williams *et al.* (2000) concluded their glyphosate review by stating that under the present and expected conditions of use, there is no possibility that this herbicide may pose a risk to human health. However, recently, other authors have reported some conflicting results (Isenring 1996, Bolognesi *et al,.* 1997; Lioi *et al.*, 1998a, 1998b; Monroy *et al.,.* 2005). Results from our studies suggest that both compounds (glyphosate and AMPA) should be re-examined in the light of changes in the present conditions of use: larger volumes than those used ten years ago and in larger areas of land cultivated with transgenic crops, resistant to glyphosate. Both compounds can interact with DNA, causing deleterious effects on genetic integrity or genomic functions. There is evidence that with the present and future conditions of glyphosate use, both compounds will be appearing as pollutants in soil and water and also as residues in food; therefore, more studies are necessary to evaluate the biological effects of these pollutants on animal, human and environmental health.

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IDENTIFICACIÓN DE TRICÓPTEROS DE ALTURA DE BOLIVIA Y PERÚ MEDIANTE EL CÓDIGO DE BARRAS DEL ADN

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ABSTRACT

Order Trichoptera includes some of the biomarker insects most studied at the genetic level because of their usefulness and distribution in freshwater systems. In the absence of morpho-anatomical features to distinguish larvae of these species of highland Peru and Bolivia, here we propose the use of sequences of the CO-I gene (DNA Barcode) for molecular taxonomic identification. This is the first study of the CO-I sequences in Trichoptera larvae of Western and Eastern Cordillera of Bolivia and Cordillera Blanca of Peru. With Kimura's two-parameter model, we identified genera Anomalocosmoecus, Cailloma and Antarctoecia. More detailed analysis of the sequenced DNA Barcode suggests the existence of a suborder and two species of Antarctoecia in the Western Cordillera of Bolivia. More studies are needed to provide further support to this hypothesis.

Key words: CO-I, molecular taxonomy, Trichoptera.

RESUMEN

El Orden Trichoptera incluye algunos de los insectos bioindicadores más estudiados a nivel genético por su utilidad y distribución en sistemas de agua dulce. Al no contar con claves morfo-anatómicas de larvas para diferenciar especies altiplánicas, proponemos el uso de secuencias del gen CO-I (código de barras del ADN) para su identificación taxonómica molecular. Se estudiaron por primera vez secuencias CO-I en larvas de tricópteros de la Cordillera Oriental y Occidental de Bolivia, y de la Cordillera Blanca de Perú. Con el modelo de dos parámetros de Kimura, se identificaron los géneros Anomalocosmoecus, Antarctoecia y Cailloma. El análisis más detallado del código de barras del ADN secuenciado sugiere la existencia de un posible suborden y dos especies del género Antarctoecia en la Cordillera Occidental de Bolivia. Más estudios son necesarios para dar mayor sustento a esta hipótesis.

Palabras clave: CO-I, taxonomía molecular, Trichoptera.

INTRODUCCIÓN

Entre los macroinvertebrados mejor estudiados y utilizados como bioindicadores de calidad de agua están las especies que pertenecen al orden Trichoptera (Bonada *et al.*, 2006; Holzenthal *et al.*, 2007; Molina *et al.,* 2008). Los tricópteros son artrópodos holometábolos de estadios inmaduros acuáticos, con alrededor de 10.000 especies descritas y distribuidas entre 45 familias (Morse, 1997), de elevada diversidad de nichos tróficos (Mackay & Wiggins, 1979), y cuyo estudio resulta ser de gran importancia en la evaluación ambiental. Sin embargo, en la mayoría de los casos, la identificación taxonómica, especialmente de larvas, no ha sido posible, debido a la falta de claves taxonómicas por región o a las similitudes morfológicas entre especímenes de este estadio. De esta manera, se han desarrollado estudios de la sistemática molecular de este orden en busca de patrones de coevolución con el fin de inferir su filogenia (Morse, 1997). El método conocido como "código de barras del ADN (CO-I)" se ha usado para estudiar la variabilidad a niveles taxonómico, filogenético e incluso filogeográfico, considerando al menos un segmento informativo (Crozier *et al.*, 1989; Hwang & Kim, 1999; Muraji *et al.* 2000; Myeres *et al.,* 2001; Cánovas, 2002; Pauls, 2004; Haro *et al.,* 2005; Smith, 2005; Audisio *et al.,* 2008; Lehrian *et al.*, 2009; Bajpai & Tewari, 2010; Salinas, 2010). No obstante, pese a los evidentes efectos del cambio climático sobre la biodiversidad en el altiplano boliviano, no existen estudios del orden Trichoptera a nivel taxonómico molecular.

En el presente trabajo, proponemos el uso de CO-I para la identificación taxonómica molecular de larvas de tricópteros en sitios de la Cordillera Oriental y Occidental de Bolivia y de la Cordillera Blanca de Perú, con el fin de caracterizar este marcador molecular para la identificación taxonómica de estos bioindicadores y aportar al conocimiento de las especies existentes.

MATERIAL Y MÉTODOS

Recolección e identificación taxonómica

Se recolectaron larvas de Tricópteros en ríos afluentes de los glaciares Mururata, Illimani (Cordillera Oriental de Bolivia), Larancagua (Cordillera Occidental de Bolivia), Huáraz y Paca (Cordillera Blanca de Perú). La identificación morfo-anatómica de dichas larvas se realizó en la Unidad de Limnología del Instituto de Ecología de la Universidad Mayor de San Andrés (La Paz, Bolivia).

Extracción y amplificación del ADN

Se extrajo material genético de las patas de las larvas optimizando el sistema comercial Wizard de Promega. Se empleó la pareja de cebadores universales LCO1490 (5'GGTCAACAAATCATAAAGATATTGG-3') y HCO2198 (5'-TAAACTTC AGGGTGACCAAAAAATCA-3') descritos por Hebert *et al.* (2003). La concentración final de los cebadores fue de 0.355 μM y los fragmentos correspondientes fueron amplificados mediante el sistema comercial de GoTaq Colorless Master Mix (Promega). El programa empleado fue: 5 min a 94°C; 30 ciclos de: 1 min a 94°C, 110 s a 51°C, 110 s a 72°C; y extensión de 10 min a 72°C.

Obtención de secuencias de ADN

Luego de aplicar una purificación alcohólica a los productos amplificados, los productos se secuenciaron empleando el cebador LCO1490 y el sistema Big Dye Terminator v3.1. (Applied Biosystems), según el protocolo del fabricante. Tras una nueva purificación alcohólica, las secuencias se obtuvieron por electroforesis capilar en un Analizador Genético AB3130 (Applied Biosystems).

Análisis de secuencias

Los resultados se introdujeron en el programa informático "Sequence Analysis" y los electroferogramas se editaron manualmente con los programas BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) y SeqA 5.2. (Applied Biosystems).

Asimismo, se introdujeron secuencias CO-I de los órdenes Mecóptera, Lepidóptera, Díptera y Trichoptera disponibles en GenBank (http://www.ncbi.nlm.nih.gov/genbank/), y se alinearon empleando ClustalW del programa Mega 4 (Tamura, *et al.*, versión 4).

Finalmente, para establecer los patrones taxonómicos moleculares, se empleó el modelo de distancia genética de dos parámetros de Kimura (Kumur & Nei, 2000) más el método Neighbor-Joining (NJ) (Saitou & Nei, 1987) de construcción de filogramas con consistencia igual a 1.000 réplicas.

RESULTADOS

Entre los 45 especímenes de larvas de tricópteros estudiados, morfo-anatómicamente, se identificaron tres géneros (Antarctoecia, Anomalocosmoecus y Cailloma) en los glaciares bolivianos Mururata, Illimani y Larancagua, y

un género (Anomalocosmoecus) en los glaciares Huáraz y Paca de Perú.

En el estudio genético, se obtuvo un fragmento de ADN de 709 pb, flanqueado por las posiciones 1490 y 2198 pb (ADNmt) para todos los especímenes estudiados. Luego de la edición y el alineamiento de las secuencias, se comparó un sub-fragmento de 636 pb con 211 sitios polimórficos entre los haplotipos de especímenes bolivianos (Fig. 1). Se identificaron ocho haplotipos de tricópteros en los especímenes de Bolivia y uno en los de Perú.

El mayor porcentaje de sustituciones de tipo transición (timinas y citosinas) observado es coherente con el patrón característico esperado del marcador mitocondrial CO-I. Dicho marcador CO-I permitió caracterizar dos clados entre las familias Limnephilidae y Lepidostomatidae (Fig. 2).

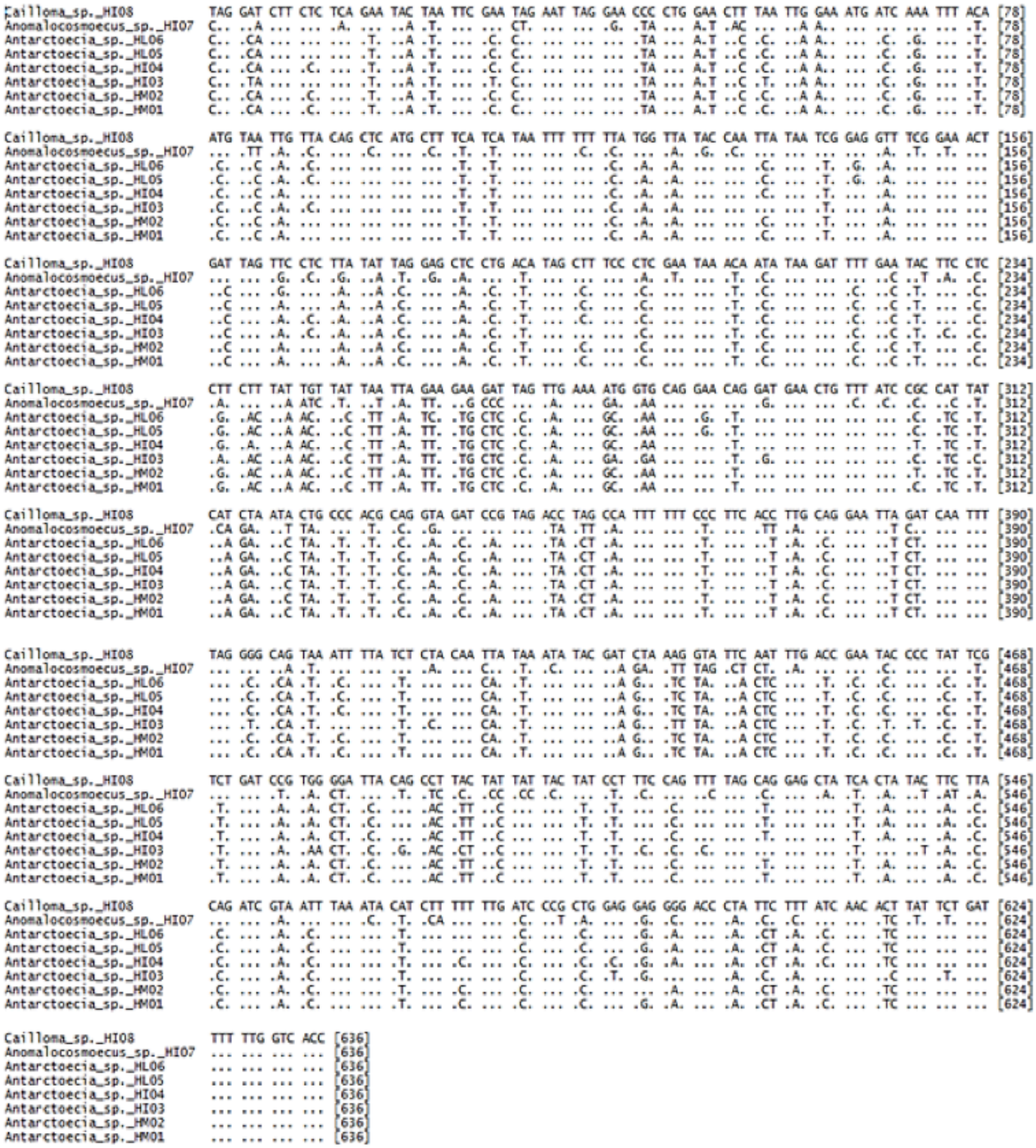

Figura 1. Secuencia nucleotídica de CO-I (636 pb) de especímenes tricópteros bolivianos;
HM = haplotipos del glaciar Mururata; HI = haplotipos del glaciar Illimani; HL = haplotipos del glaciar Larancagua

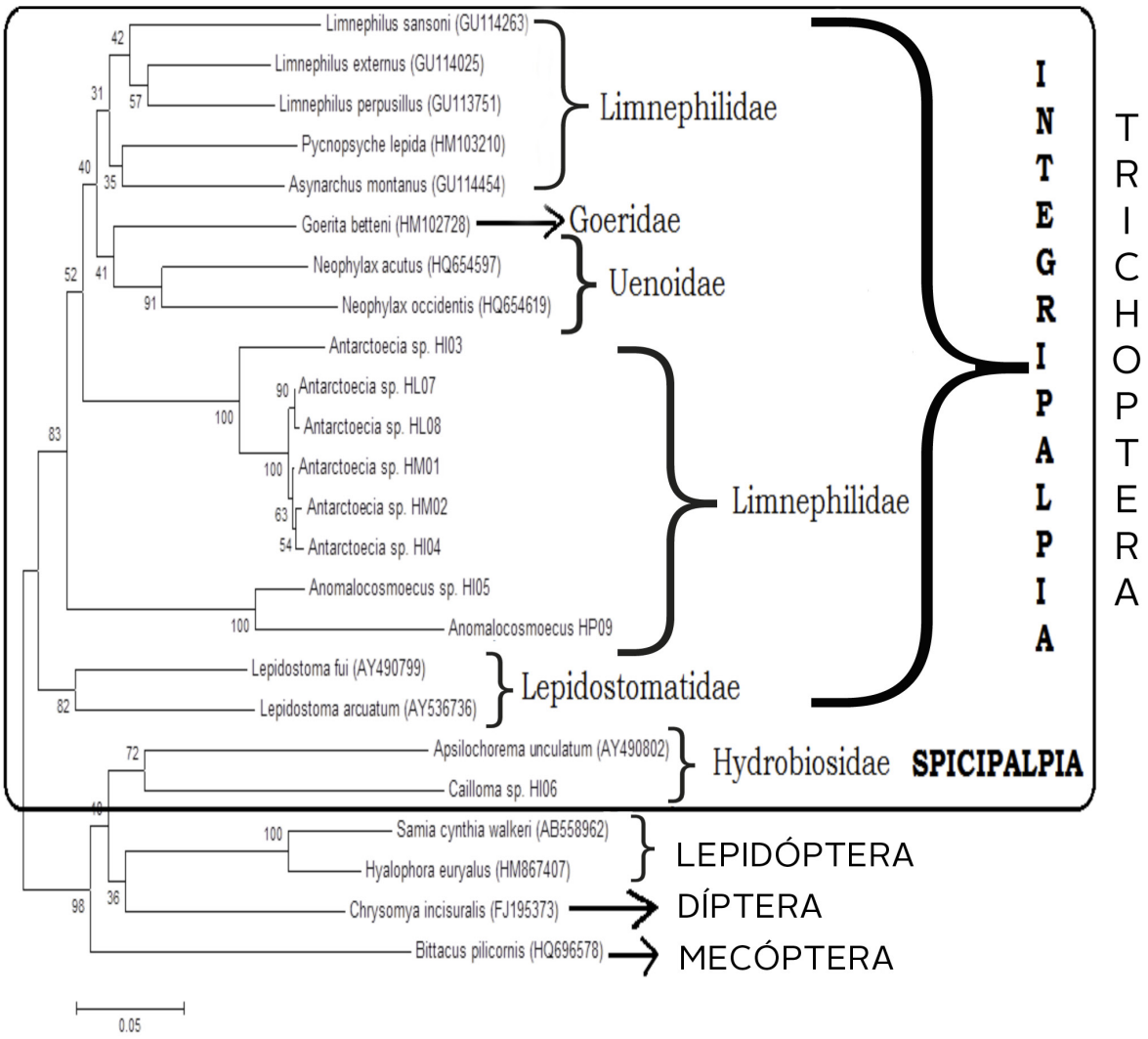

Figura 2. Árbol taxonómico molecular del orden Trichoptera en base al modelo de dos parámetros de Kimura (Kumur & Nei, 2000). Ilustra la capacidad del fragmento amplificado del gen CO-I (439pb) para la diferenciación taxonómica a nivel de género, familia, suborden y orden, así como la capacidad de diferenciación filogeográfica, donde HM01, HM02 = Mururata; HI03, HI04, HI05, HI06 = Illimani; HL07, HL08 = Larancagua; HP09 = Perú. Número de acceso del GenBank entre paréntesis.

DISCUSIÓN

En relación a la caracterización de dos clados entre las familias Limnephilidae y Lepidostomatidae, dentro de la primera se observaron tres subclados a nivel de género: 1) Anomalocosmoecus, 2) Antarctoecia, y 3) Limnephilus, Neophylax y otros que son propios de Apsinachus. Este último subclado contiene las familias limnefílidas Goeridae y Uenoidae las que al parecer, separan a los limnefílidos según su distribución, es decir entre el Norte y Sur de América. *Goerita betteni* (Goeridae) es del este y *Neophylax acutus* y *Neophylax occidentis* del oeste de Estados Unidos, aunque también existen otras especies en el Asia. Quizás estas especies fueron identificadas como miembros de otra familia por su distribución y algunas características morfo-anatómicas, pero pertenecen a la familia Limnephilidae, de acuerdo a la clasificación de Vineyard y Wiggins 1987. Nozaki *et al.* (2000) mencionan que la clasificación de las familias Uenoidae, Goeridae y Limnephilidae es muy incierta cuando la misma se basa en la anatomía de larvas, mientras que Zhou *et al.* (2011), mediante un estudio del CO-I, fortalecieron la existencia de las familias Uenoidae y Goeridae. Por tanto, se refuerza la necesidad de estudiar más a fondo el orden Trichoptera en Bolivia.

El dendograma obtenido en el presente trabajo refleja no sólo la taxonomía tradicional sino también la distribución geográfica de tricópteros. Por la disponibilidad de la secuencia del espécimen peruano (HP09), únicamente se emplearon 439 pb para la construcción del dendograma. Pese a ello, las pruebas con dicho espécimen y sin él, ampliando el fragmento a 636 pb presentan el mismo árbol taxonómico, por tanto la misma información taxonómica molecular que se observa en la Fig. 1.

Observando el polimorfismo de este marcador a nivel de especie, *Lepidostoma* contiene alrededor de 90 nts. diferentes entre una especie y otra. En *Drusus,* otra región del CO-I muestra que éste presenta una diferencia de 53 nts., y que la distancia genética entre especies oscila entre 0.030 – 1.000. En el presente estudio, *Antarctoecia* HI03 y HI04 presentan 35 nts. diferentes y una distancia igual a 0.0585. Por su parte, Smith (2005) identificó diferencias de un aminoácido entre dos especies cercanas de chicharras, en el presente estudio se identificaron diferencias aminoacídicas en las posiciones 92 y 161, donde se observan mutaciones de arginina a serina y de alanina a treonina. Sin embargo, las especies de *Lepidostoma* presentan cinco aminoácidos diferentes y *Anomalocosmoecus* ocho. Tanto el modelo de distancia genética como el valor de consistencia empleado revelan la utilidad del marcador CO-I en especies de tricópteros bolivianos. Esto permite postular posible presencia de especies o subespecies diferentes del género Antarctoecia, pero no asegura que efectivamente se trate de especies distintas, ya que el valor de divergencia no es tan alto como en otros individuos. Si bien Smith (2005) detectó sólo una diferencia aminoacídica, el comportamiento en este género y en este orden podría ser diferente, y por ello los resultados se deben contrastar con un estudio morfo-anatómico. Por otro lado, el patrón de los clados de Antarctoecia y Anomalocosmoecus es similar, y morfo-anatómicamente se identificó una especie distinta de Anomalocosmoecus en Perú, por lo que estos individuos podrían ser especies diferentes.

CONCLUSIONES Y RECOMENDACIONES

El presente trabajo es el primer estudio exploratorio de tricópteros bolivianos a nivel molecular. Se empleó una región del código de barras del ADN, que resultó ser efectivo para la clasificación de los especímenes estudiados a nivel de sub-orden, género y especie (Pauls, 2004; Smith, 2005; Haro *et al.,* 2005; Bajpain & Tewari, 2010). Aunque esta región es conservada en su secuencia aminoacídica, presenta diferencias en las posiciones de sus aminoácidos de una especie a otra, lo que respalda su posible uso como marcador taxonómico a nivel de especie, y muestra una estructura que puede explicarse y cotejarse con la distribución geográfica de especímenes, por lo que se sugiere su uso también para estudios filogeográficos de estos insectos en el altiplano boliviano.

Se recomienda realizar estudios morfo-anatómicos complementarios para verificar la presencia de las aparentes especies y subespecies caracterizadas genéticamente en el presente estudio para los géneros Antarctoecia, Anomalocosmoecus y Cailloma.

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