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**DEPARTAMENTO DE GENÉTICA**

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**Marcela Beatriz Ayala Benítez**

**Córdoba, 2015**

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AUTOR: *Marcela Beatriz Ayala Benítez*

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Trabajo realizado por Marcela Beatriz Ayala Benítez,
Ingeniera Agrónoma para optar por el grado de Doctora.

Directores:
Dr. Juan Bautista Álvarez Cabello
Dr. Carlos Guzmán García

**Córdoba, 2015**

**TÍTULO DE LA TESIS:** CARACTERIZACIÓN MOLECULAR DE VARIANTES ALÉLICAS PARA LOS LOCI *GLU-1*, *WX* Y *HA* EN ESPECIES DE TRIGO HEXAPLOIDE ABANDONADAS O INFRAUTILIZADAS

**DOCTORANDO/A:** MARCELA BEATRIZ AYALA BENITEZ

## INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

La Tesis ha presentado un desarrollo coherente con las previsiones realizadas en su momento. Dentro de la misma se ha realizado una estancia breve en el Centro Internacional de Mejoramiento de Maíz y Trigo (CIMMYT), situado en México, donde se llevaron a cabos algunos de los análisis de los trabajos desarrollando en esta Tesis.

Los trabajos desarrollados y que conforman esta Tesis han permitido la caracterización morfológica de materiales tradicionales de Andalucía, junto con la detección y caracterización de nuevas variantes alélicas para los loci motivo de estudio en tres colecciones de trigos abandonados o infrautilizados: trigos criollos mexicanos, variedades tradicionales u obsoletas de trigos harineros andaluces, y materiales de tres subespecies de trigo hexaploide (cabezorro, macha y indio enano). La variación detectada ha incluido también variantes de baja presencia en materiales modernos, lo que puede ser muy útil para la inclusión de nuevos parentales en los programas de mejora de la calidad de trigo. Esto permitirá el desarrollo a medio-largo plazo de nuevos cultivares de trigo que presenten nuevas propiedades.

En base a este desarrollo, se ha optado por la presentación de esta Tesis como compendio de publicaciones, comprendiendo un total de 4 publicaciones (capítulos I al IV del documento), de los cuales actualmente se encuentra publicada una en la revista «*Euphytica*», junto con dos en prensa en la revista «*Theoretical and Applied Genetics*» y «*Genetic Resources and Crop Evolution*», todas ellas incluidas en algunas de las categorías del «*Science Citation Index*». En cuanto a la otra, en el momento de la emisión de este informe, está en revisión en la revista «*Genetic Resources and Crop Evolution*».

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 19 de octubre de 2015

Firma del/de los director/es

Fdo.: Juan Bautista Alvarez Cabello

Fdo.: Carlos Guzmán García

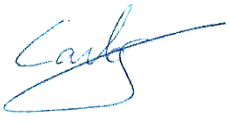
**Juan Bautista Álvarez Cabello**, Profesor Titular de la Universidad de Córdoba y **Carlos Guzmán García**, Laboratorio de Química y Calidad de Trigo, Programa Global de Trigo, Centro Internacional de Mejoramiento de Maíz y Trigo (CIMMYT), Texcoco, México.

**INFORMAN:**

Que el trabajo titulado «**Caracterización molecular de variantes alélicas para los loci *Glu-1*, *Wx* y *Ha* en especies de trigo hexaploide abandonadas o infrautilizadas**», realizado por Doña Marcela Beatriz Ayala Benítez, bajo su dirección se considera ya finalizado y puede ser presentado para su exposición y defensa como Tesis Doctoral en el Departamento de Genética de la Universidad de Córdoba.

Firmado en Córdoba, a 26 de octubre de 2015

Fdo.: Juan Bautista Alvarez Cabello



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**Marcela Beatriz Ayala Benítez** ha sido beneficiaria de una beca predoctoral del Programa de Becas de ITAIPÚ Binacional – Paraguay y una ayuda para la estancia predoctoral del INBIO (Instituto de Biotecnología Agrícola) – Paraguay.

*A mis padres, hermanos y sobrinos.*

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Sólo me queda decirles a todos ustedes **Aguyje** (Gracias en guaraní).

## LISTADO DE PUBLICACIONES OBTENIDAS Y ESTATUS DE LAS MISMAS A LA FECHA DE FIRMA DEL PRESENTE DOCUMENTO

1. Ayala M, Guzmán C, Alvarez JB, Peña RJ (2013) Characterization of genetic diversity of puroindoline genes in Mexican wheat landraces. *Euphytica* 190:53 - 63.

2. Ayala M, Guzmán C, Peña RJ, Alvarez JB (2015) Genetic diversity and molecular characterization of puroindolines (*Pina-D1* and *Pinb-D1*) genes in bread wheat landraces from Andalusia (Southern Spain). *Genetic Resources and Crop Evolution* (under review).

3. Ayala M, Guzmán C, Peña RJ, Alvarez JB (2015) Diversity of phenotypic (plant and grain morphological) and genotypic (glutenin alleles in *Glu-1* and *Glu-3* loci) traits of wheat landraces (*Triticum aestivum*) from Andalusia (Southern Spain). *Genetic Resources and Crop Evolution*. (en prensa, DOI 10.1007/s10722-015-0264-0).

4. Ayala M, Alvarez JB, Yamamori M, Guzmán C (2015) Molecular characterization of waxy alleles in three subspecies of hexaploid wheat and identification of two novel *Wx-B1* alleles. *Theoretical and Applied Genetics* 128: 2427 – 2435.

**Nota:** A fin de establecer una coherencia formal a lo largo del presente documento, se han uniformado las referencias y se han editado los trabajos originales, eliminando de los mismos el apartado de referencias, el cual ha sido agrupado al final del documento.

# ÍNDICE

**RESUMEN**

En esta Tesis Doctoral se han evaluado una serie de colecciones de trigos hexaploides de diversa procedencia geográfica. Así, se han utilizado variedades criollas mexicanas, junto con variedades tradicionales u obsoletas de Andalucía, de trigo harinero (*T. aestivum* ssp. *aestivum*). A estos materiales se han añadido las colecciones de trigo cabezorro o club (*T. aestivum* ssp. *compactum*), enano de la India (*T. aestivum* ssp. *sphaerococcum*) y trigo macha (*T. aestivum* ssp. *macha*). La caracterización de alguna de estas colecciones ya había sido iniciada en trabajos previos de nuestro grupo de investigación. Por ello, en el caso de las variedades criollas mexicana sólo se evaluó la textura y la variación para puroindolinas. Los resultados mostraron que los alelos presentes en estos materiales para ambas puroindolinas ya habían sido previamente descritos. En las variedades locales de Andalucía, para las mismas proteínas, también se obtuvo un resultado similar. Sin embargo, es de destacar que algunos de ellos, han sido considerados raros debido a su escasa frecuencia en los materiales evaluados por otros autores. Además sobre la misma colección de variedades locales de Andalucía se caracterizaron para la morfología de la planta, espiga y grano y para la composición de HMWGs y LMWGs. Además de la amplia variación para los caracteres morfológicos detectados en estas variedades locales, el análisis para HMWGs y LMWGs mostró una gran variación. La variación alélica en el *Glu-1* y *Glu-3* mostró un claro riesgo de erosión genética debido a la baja frecuencia de algunos alelos. Otro de los factores importantes en la calidad de la harina es la composición del almidón del grano de trigo, en donde varios estudios han contribuido al catálogo de alelos waxy disponibles para los mejoradores, aunque sigue la búsqueda de nuevos alelos de estas y otras proteínas relacionadas con la calidad de la harina. En el último capítulo se describe la caracterización de

dos alelos nuevos *Wx-B1* en una colección de trigo macha, trigo enano de la India y trigo cabezorro o club. Estas mutaciones eran nuevas y catalogadas provisionalmente como *Wx-B1k* y *Wx-B1m*, respectivamente, y podrían utilizarse para ampliar la variabilidad genética de este gen. En conclusión podemos decir que la variabilidad encontrada en los materiales evaluados podría ser utilizada tanto para su recuperación como cultivo *per se* como para su uso en programas de mejoramiento, además de ofrecer rasgos de calidad deseados para la elaboración de los alimentos tradicionales.

**ABSTRACT**

In this Doctoral Thesis different collections of hexaploid wheat geographically dispersed have been evaluated. So, Mexican landraces along with traditional Andalusian or obsolete varieties of bread wheat (*T. aestivum* ssp. *aestivum*) have been used. Moreover, club wheat (*T. aestivum* ssp. *compactum*) Indian dwarf (*T. aestivum* ssp. *sphaerococcum*) and macha wheat (*T. aestivum* ssp. *macha*) are also included in the analysed material. Characterization of some of these collections was initiated in previous studies of our research group. For this reason, in the case of Mexican landraces, only the hardness and the variation for puroindolines were evaluated. The results showed that the alleles detected for both puroindolines in these materials had been previously described. Similar results were obtained for these same proteins in the Andalusian bread wheat landraces. However, some of these alleles have been considered rare because of its low frequency in the materials evaluated by other authors. The same collection of landraces from Andalusia was also characterized for plant, spike and grain morphology, together with the HMWGs and LMWGs composition. Additional to the wide variation from morphological traits showed by these landraces, the HMWGs and LMWGs analysis showed great variation. Allelic variation in the *Glu-1* and *Glu-3* showed a clear risk of genetic erosion due to the low frequency of some alleles. Another important quality factor of flour is starch composition of the wheat kernel, where several studies have contributed to the catalogue of waxy alleles available for breeders, while still finding new alleles of these and other related proteins to flour quality. In the last chapter it was described the characterization of two new *Wx-B1* alleles in a collection of macha wheat, Indian dwarf wheat and club wheat. These mutations were provisionally classified as *Wx-B1k* and *Wx-B1m*, respectively, and could be used to

broaden the genetic variability of this gene. In conclusion we could say that the variability found in the studied materials could be used both for recovery as a per se crop and to be used in breeding programs, as well as offering quality features desired for the preparation of traditional foods.

# INTRODUCCIÓN

**Generalidades**

El nombre genérico de trigo se aplica a diferentes especies y subespecies del género *Triticum*. Todas ellas son plantas anuales cuya clasificación botánica completa es la siguiente:

Reino: *Plantae*

División: *Magnoliophyta*

Clase: *Liliopsida*

Subclase: *Commelinidae*

Orden: *Poales*

Familia: *Poaceae*

Género: *Triticum*

El trigo es uno de los tres cultivos, junto con el maíz y el arroz, con mayor producción y superficie cultivada a nivel mundial. Esto es debido principalmente a que posee una gran adaptabilidad agronómica, que permite su cultivo en prácticamente todas las latitudes, y a que su grano es de fácil almacenamiento y conservación. A ello se suma que las características de su harina permiten la producción de una gran variedad de alimentos con una excelente palatabilidad.

Según datos de FAO (2015), la estimación de producción del trigo en el mundo se situaría en torno a los 720 millones de toneladas para el presente año (Tabla 1). Esto supondrá una reducción del 1% (8 millones de toneladas) con respecto a 2014, principalmente debido a una productividad más baja de lo esperado en la Unión Europea (UE) y la Federación de Rusia, donde se prevé que los rendimientos vuelvan a los niveles promedio tras haber alcanzado valores excepcionalmente elevados en 2014.

**Tabla 1.** Principales países productores de trigo

| País | Toneladas métricas |
|---|---|
| China | 126.000.000 |
| India | 94.500.000 |
| Estados Unidos | 56.000.000 |
| Rusia | 55.000.000 |
| Unión Europea | 147.000.000 |
| España | 7.597.900 |
| Resto del Mundo | 241.500.000 |
| Canadá | 30.000.000 |
| Australia | 26.000.000 |
| Pakistán | 25.500.000 |
| Ucrania | 22.000.000 |
| Turquía | 21.000.000 |
| Irán | 13.000.000 |
| Kazajstán | 13.500.000 |
| Argentina | 12.000.000 |
| Egipto | 8.500.000 |
| Uzbekistán | 7.500.000 |
| Otros | 62.500.000 |
| Total | 720.000.000 |

Fuente: FAOSTAT 2015.

**Origen y estructura genética**

El cultivo de trigo se remonta a hace unos 10.000 años en la zona del Creciente Fértil, en la parte sur oriental de la actual Turquía. El cultivo se extendió a Próximo Oriente hace unos 9.000 años, cuando el trigo hexaploide hizo su primera aparición (Feldman 2001).

El trigo está constituido por un complejo poliploide, formado por especies tanto silvestres como cultivadas (Fig. 1). Según su nivel ploídico, estas especies se pueden clasificar en:

- Diploides: con un solo genoma (**A**) constituido por 7 parejas de cromosomas (2n = 2× = 14). En este grupo se encuentran el einkorn o escaña (*Triticum monococcum* L. ssp. *monococcum*), y *T. urartu* Thum. ex Gandil.

- Tetraploides: con dos genomas diferentes (**AB**), y un total de 14 parejas de cromosomas (2n = 4× = 28). A este grupo pertenecen todos los trigos duros conocidos.
- Hexaploides: con tres genomas diferentes (**ABD**) y 21 parejas de cromosomas (2n = 6× = 42). En este grupo se encuentra el trigo harinero utilizado actualmente.

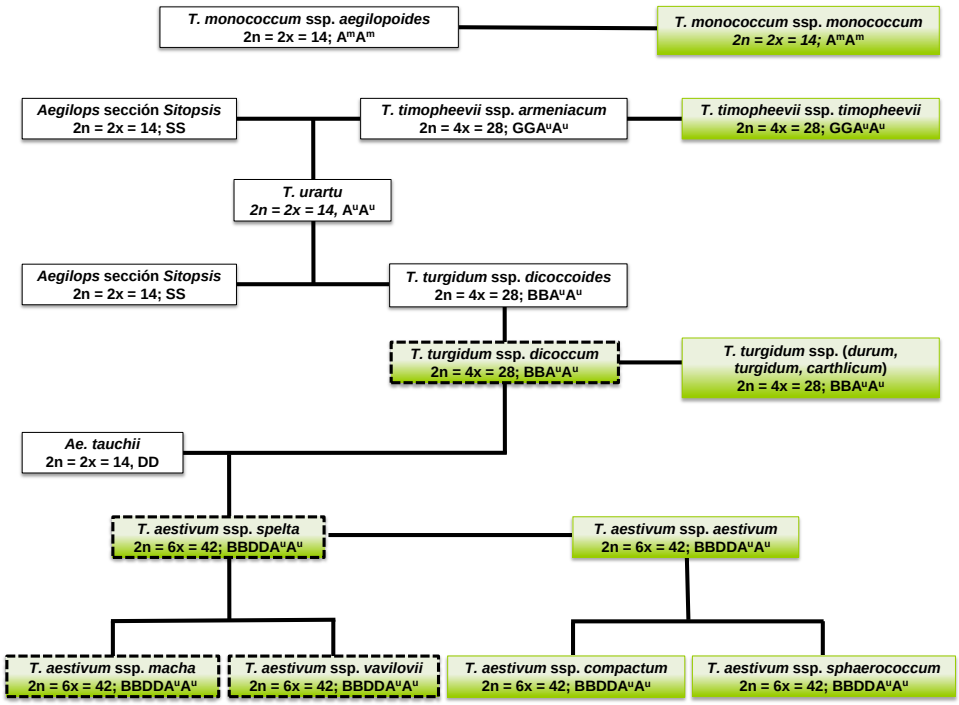


**Figura 1.** Origen y evolución del trigo. Las especies en verde son o han sido en algún momento cultivadas, y las que aparecen con línea discontinua son de grano vestido.

La primera especie cultivada fue la escaña (*T. monococcum* ssp. *monococcum*, $\mathbf{A^mA^m}$), que procede de la domesticación de *T. monococcum* ssp. *aegilopoides* Link em. Thell. (syn. *T. boeoticum* Boiss.), en la zona del Creciente Fértil. Sin embargo, ha sido otra especie derivada del mismo ancestro, en este caso silvestre, *T. urartu* ($\mathbf{A^uA^u}$), la que ha sido propuesta como donadora del genoma **A** en las especies poliploides de trigo (Dvorak 1988). Los análisis

genéticos sugieren que *T. urartu* pudo participar en los dos eventos evolutivos que generaron los principales trigos tetraploides (Dvorak y Zhang 1990; 1992). En ambos eventos, los híbridos resultantes sufrieron procesos de duplicación cromosómica mediante endomitosis natural. En el primero de estos eventos, el cruzamiento de *T. urartu* con una especie de la sección *Sitopsis* del género *Aegilops*, probablemente *Ae. speltoides* Taush (2n = 2× = 14, **SS**), si bien no se descarta un origen polifilético que involucra a varias de estas especies (Sarkar y Stebbins 1956), dio lugar al emmer silvestre (*T. turgidum* ssp. *dicoccoides* Körn. ex Asch. & Graebner em. Thell.; 2n = 4× = 28, **BBA$^{u}$A$^{u}$**), a partir del cual por domesticación se obtuvo el emmer cultivado o escanda menor (*T. turgidum* spp. *dicoccum* Shrank em. Thell.), del cual derivan el resto de los trigos tetraploides destacando el trigo duro (*T. turgidum* ssp. *durum* Desf. em. Husn.). En el otro evento, Kilian et al. (2007) identificaron a *Ae. speltoides* como la especie con la cual se cruzó *T. urartu* para generar *T timophevii* ssp. *armeniacum* Jakubz. em. Slageren (2n = 4× = 28, **GGA$^{u}$A$^{u}$**), cuya forma domesticada *T. timophevii* ssp. *timophevii* se restringe al occidente de Georgia.

Los trigos hexaploides se originaron por la hibridación y posterior duplicación cromosómica entre *T. turgidum* ssp. *diccoccum* y *Ae. tauschii* Coss., identificado como donador del genoma **D** (McFadden y Sears 1946; Kerber y Rowland 1974), dando lugar a *T. aestivum* ssp. *spelta* L. em. Thell. (2n = 6× = 42 **BBDDA$^{u}$A$^{u}$**). Esta hibridación debió producirse en un campo de cultivo de emmer en la zona del Mar Caspio, hábitat natural de *Ae. tauschii*, lo que viene apoyado en la inexistencia de especies de trigo silvestres hexaploides. A partir del espelta acabaría originándose el trigo común o harinero (*T. aestivum* L. ssp. *aestivum*), siendo la especie más importante del género *Triticum* en la actualidad.

**Usos tradicionales y actuales del trigo**

La elaboración del pan a partir del trigo es uno de los usos más importantes y conocidos desde la antigüedad, así como la elaboración de pastas y cervezas. Las primeras referencias escritas se remontan al 2.600 a.C., aunque los restos arqueológicos sugieren que la elaboración de pan podría remontarse hasta el 4.000 a.C. En cuanto al esponjamiento del pan, los egipcios fueron los primeros en utilizar la levadura para este fin (Harlan 1981). En esa época se usaba el pan como un emblema de estatus social: las clases humildes comían un pan tosco y de baja calidad, mientras que los sacerdotes y los altos cargos del estado, incluyendo al Faraón, consumían panecillos realizados con las mejores harinas (Harlan 1981; Pomeranz 1987). Estos conocimientos fueron transmitidos a las diversas culturas del Egeo hasta llegar a los antiguos griegos, quienes también los transmitieron a los romanos. A partir de estos últimos, para los cuales tuvo incluso un valor cultural y religioso, el trigo comienza a tener la importancia en la alimentación que hoy conocemos.

Con el paso del tiempo y la llegada de la Revolución Industrial, se fueron utilizando nuevas técnicas de molturación, amasado y horneado. A esto hubo que sumar el gran aumento de la población, lo que provocó a su vez la búsqueda de variedades más rendidoras en el campo de cultivo. Además, la mecanización de los procesos de panificación en la industria demandó variedades cuya harina formase una masa fuerte que ofreciera tolerancia al amasado y sobreamasado mecánico. Esta demanda de variedades caracterizadas principalmente por una alta producción y tolerancia al sobreamasado mecánico se ha mantenido hasta nuestros días, y ha llevado a la sustitución de las numerosísimas variedades locales por unas pocas variedades modernas muy productivas y adaptadas a la industria panificadora y semolera.

La forma de elaboración del pan ha continuado evolucionando y diversificándose hasta nuestros días, llegando a tener la gran variabilidad que disponemos actualmente como son: el pan sin levadura, pan de masa ácida, pan sin gluten, pan integral y miles de tipo de pan para satisfacer el gusto de los consumidores. Además, en la actualidad, el trigo tiene muchos otros usos en la industria alimentaria, utilizándose para la elaboración de extruidos, galletas, tallarines orientales, productos de repostería y pastas. Estos son consumidos por la gente de todas las culturas y religiones (Bushuk 1998). También se ha empezado a usar en productos no alimenticios como cosméticos e incluso como cultivo energético para la producción de bioetanol o biomasa (Bell et al. 1995).

**Calidad harino-panadera del trigo**

Se puede definir la calidad del trigo como la capacidad de una variedad de producir una harina o sémola adecuada para un producto específico. Por tanto, es un parámetro variable que va a depender de las preferencias del consumidor, del producto que se quiera elaborar (no se necesitan las mismas características de calidad para elaborar pan o galletas, por ejemplo) y del proceso que se va a utilizar en la elaboración de dicho producto.

La calidad está determinada por las características físico-químicas del grano y de las harinas, que dependen en gran medida de factores genéticos intrínsecos a la variedad o cultivar utilizado, si bien están también sujetas a la influencia de factores ambientales (suelo, clima, técnicas de cultivo y almacenamiento) y de procesamiento (Van der Veen y Palmer 1997).

El grano del trigo está compuesto por proteínas (7-18%), lípidos (1,5-2%) y carbohidratos (60-75%), y otros compontes menores como vitaminas y trazas de minerales (Aykroyd y Doughty 1970). Las proteínas y los hidratos de carbono, especialmente almidón, tienen influencias considerables en tres

características del grano estrechamente vinculadas a la calidad requerida en el trigo para sus diferentes usos.

- Las puroindolinas, responsables de la dureza del grano;
- Proteínas de reserva del endospermo (gliadinas y gluteninas), responsables de la formación del gluten durante el amasado de la harina, y
- Las proteínas sintetizadoras del almidón.

**La dureza del grano: puroindolinas**

La dureza o textura del grano es el principal determinante para la clasificación y uso final del trigo en el mercado mundial de grano. De acuerdo a esta característica, los trigos se clasificarían en extraduros, que comprende el trigo duro o semolero, mientras que el trigo harinero es clasificado como blando o *soft,* y tenaz-duro o *hard* (Morris y Rose 1996).

La dureza del grano está controlada por el locus *Ha*, situado en el brazo corto del cromosoma 5D del trigo (Symes 1965; Morris 2002). Es un locus complejo formado por 10 genes estrechamente ligados (Fig. 2), de los que tres (*Pina-D1*, *Pinb-D1* y *Gsp-1*) han sido asociados a la dureza del grano en mayor o menor medida (Gautier et al. 1994; Rahman et al. 1994). Dos de estos genes, *Pina-D1* y *Pinb-D1,* codifican para las puroindolinas, dos proteínas íntimamente ligadas con esta propiedad del grano (Morris et al. 1994; Morris 2002; Hogg et al. 2004). Cuando ambos genes están en su forma silvestre, la textura del grano es blanda o suave. Sin embargo, cuando están presentes mutaciones en uno u otro de los genes, la textura del grano es dura (Gautier et al. 1994; Giroux y Morris 1997; Lillemo y Morris 2000; Morris 2002; Chen et al. 2006). El extremo de esta circunstancia lo representa el trigo duro, donde la textura del grano es muy dura siendo la consecuencia de la deleción de ambos genes (Gautier et al. 2000), proceso acontecido durante la generación de los trigos tetraploides por la eliminación parcial del

locus *Ha* en los genomas A y B (Li et al. 2008). La hibridación posterior con *Ae. tauschii* permitió la recuperación del carácter blando en los trigos hexaploides por la aportación de los dos genes procedentes del genoma D.

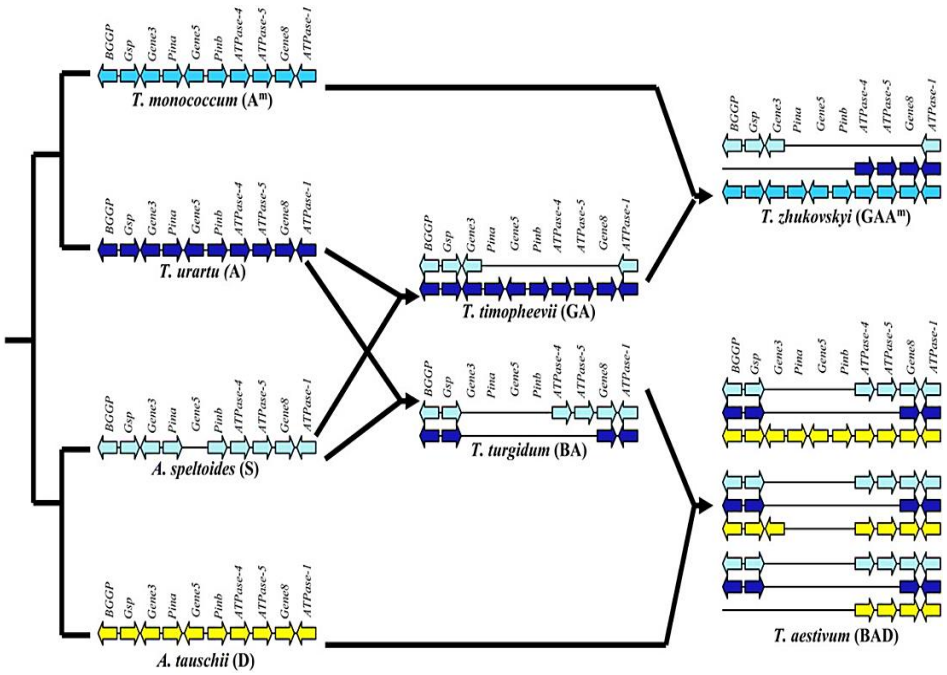


**Figura 2.** Esquema y evolución del locus *Ha* en el complejo trigo (Li et al. 2008).

La dureza del grano está determinada por el modo en que se empaquetan los gránulos de almidón en el endospermo. En los trigos con textura suave (*soft*), la adhesión entre los gránulos de almidón y la matriz proteica en el endospermo es más débil que en los trigos con textura dura (*hard*). Durante la molienda, los planos de fractura en los trigos de textura suave se producen entre los gránulos de almidón y la matriz proteica, mientas que en los trigos de textura dura, los planos de fractura se producen dentro de los gránulos (Peña 2002). Por tanto, en función de la compactación de estos gránulos de almidón se producirá mayor o menor nivel de almidón dañado, que está relacionado con la absorción de mayor cantidad de agua.

Una pequeña cantidad de almidón dañado es deseable en harinas para la fabricación de galletas y repostería porque le confiere poca absorción de agua, lo que permite una correcta cocción y expansión de las galletas en el horneado. En el caso de la elaboración del pan, se requiere mayor cantidad del almidón dañado, para que la masa tenga una alta absorción de agua que permita su correcto desarrollo durante la fermentación y una mayor frescura del producto (Peña 2002).

**Proteínas de reserva**

El gluten ha sido definido como «la estructura viscoelástica que queda tras el lavado del almidón de una masa formada con harina de trigo» (Miflin et al. 1983). Contiene alrededor de 80% de proteínas, 5-10% de lípidos, almidón residual, carbohidratos y proteínas insolubles en agua (Nierle y El Baya 1990). Las proteínas de reserva, también llamadas prolaminas debido a su alto contenido en prolina y glutamina, están constituidas por dos clases principales: gliadinas y gluteninas.

Las gliadinas son proteínas monómericas con un peso molecular comprendido entre 30 y 80 kDa, unidas mediante puentes de hidrógeno e interacciones hidrofóbicas. Cuando se fraccionan electroforéticamente en medio ácido, presentan cuatro grupos en función de su movilidad con respecto al cátodo: ω-, γ-, β- y α-gliadinas. El análisis genético ha establecido que, mayoritariamente, cada uno de estos grupos está sintetizado por genes localizados en el brazo corto de los cromosomas homeólogos 1 y 6. El loci *Gli-1* del grupo de cromosomas homeólogos 1 (*Gli-A1*, *Gli-B1* y *Gli-D1*) controla las γ- y ω-gliadinas; el loci *Gli-2* (*Gli-A2*, *Gli-B2* y *Gli-D2*) del grupo homeólogo 6 controla las α/β-gliadinas (Metakovsky et al. 1984; Ciaffi et al. 1997; Wrigley et al. 2006).

Las gliadinas no sólo han sido estudiadas por su importancia en las propiedades viscoelásticas del gluten al ser las responsables de la cohesividad

y extensibilidad de la masa, sino por su elevado nivel de polimorfismo que las convierte en un interesante marcador para analizar la variación genética, ya que el patrón electroforético de cada variedad suele ser único (Lafiandra et al. 1990), pudiendo usarse para la identificación de cultivares en trigo (Bushuk y Zillman 1978; Nevo y Payne 1987).

Las gluteninas son proteínas poliméricas con un amplio rango en cuanto a peso molecular, encontrándose unidas covalentemente mediante puentes de disulfuro (S-S). De acuerdo a su movilidad en electroforesis desnaturalizante a pH básico sobre geles de poliacrilamida (SDS-PAGE), se pueden diferenciar dos tipos de subunidades: subunidades de alto peso molecular (HMWGs) y subunidades de bajo peso molecular (LMWGs) (Payne et al. 1984). Estas proteínas en su conjunto confieren la elasticidad o tenacidad de la masa.

En trigo harinero, las HMWGs, cuyo peso molecular está entre 80-140 kDa, están codificadas en los loci *Glu-A1*, *Glu-B1* y *Glu-D1* situados en el brazo largo de cada uno de los cromosomas del grupo homeólogo 1 (Bietz et al. 1975; Lawrence y Shepherd 1981; Payne 1987). Cada locus consiste en dos genes estrechamente ligados los cuales codifican para dos tipos de subunidades con diferente peso molecular: subunidades tipo *x*, con mayor peso molecular, y subunidades tipo *y*, más pequeñas (Harberd et al. 1986). Ambas proteínas tienen una estructura similar formada por un dominio amino terminal (N-terminal), un dominio central repetitivo y un dominio carboxilo terminal (C-terminal) -Fig. 3-. La mayoría de las subunidades tipo *x* poseen cuatro residuos de cisteína (tres en el dominio N-terminal y uno en el C-terminal); mientras que la mayoría de las subunidades tipo *y* poseen siete residuos de cisteína: cinco en el dominio N-terminal, uno en el repetitivo y otro en el C-terminal (Shewry et al. 1995). El tamaño de los dominios N- y C-terminal está muy conservado entre las diferentes HMWGs, mientras la longitud del dominio repetitivo puede sufrir grandes variaciones, estando

asociadas estas modificaciones del dominio central a la variación alélica detectada (Shewry et al. 1995, 2002).

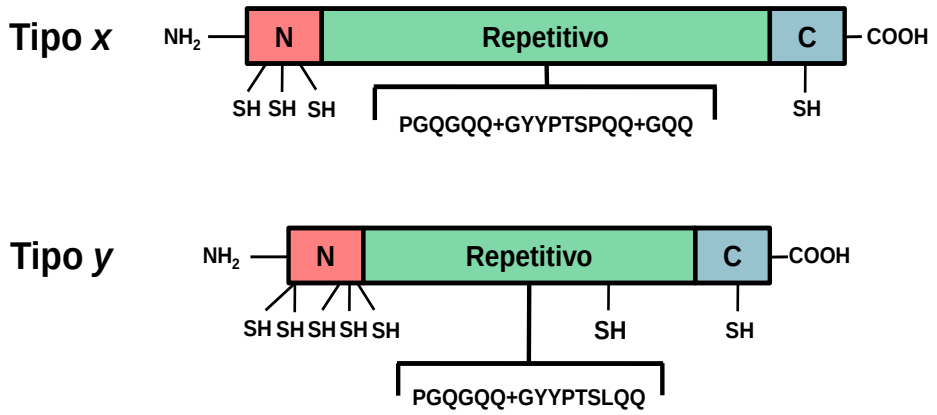


**Figura 3.** Estructura de las subunidades de tipo *x* e *y*. Se indican los motivos repetitivos del dominio central y los residuos de cisteína (-SH).

En algunos casos se produce la ausencia de estas subunidades debido al silenciamiento de los genes codificantes. El locus *Glu-A1* puede no codificar ninguna subunidad o una del tipo *x*; el tipo *Glu-B1* puede codificar sólo la subunidad *x* o ambas y el locus *Glu-D1* codifica generalmente ambas subunidades (Lawrence y Shepherd 1981; Payne et al. 1987; Werner et al. 1992).

Las LMWGs, por su parte, presentan pesos moleculares entre 30-50 kDa, estando codificadas por los loci *Glu-A3*, *Glu-B3* y *Glu-D3*, situados en el brazo corto de los cromosomas del grupo homeólogo 1 (Singh y Shepherd 1988; Liu 1995). Estos loci están estrechamente ligados con los loci *Gli-1* que codifican las γ- y ω-gliadinas (Singh y Shepherd 1988; Pogna et al. 1990). Debido a su complejidad, los estudios realizados sobre estas subunidades han sido considerablemente más escasos, si bien algunos estudios han permitido desentrañar los diferentes alelos que las conforman (Nieto-Taladriz et al. 1997; Aguiriano et al. 2008).

La relación de las proteínas de reserva con la calidad de la harina o con la sémola empleada en la elaboración de productos las convierten en una herramienta útil para mejorar la calidad, a la vez que el elevado polimorfismo las hacen candidatas a ser un buen marcador molecular para el análisis de la diversidad genética.

**El almidón y las proteínas waxy**

El almidón es el principal carbohidrato de reserva sintetizado por las plantas superiores. En el trigo, constituye la mayor parte del endospermo del grano, con aproximadamente el 65% de su peso seco (Hucl y Chibbar 1996), y juega un importante papel en la apariencia, estructura y otras características de los productos alimentarios derivados del mismo.

El almidón está formado por dos componentes estructurales diferentes: la amilosa (20-30%) y la amilopectina (70-80%) (Kuroda et al. 1989). La primera es una molécula lineal formada por residuos de D-glucosa unidos por enlaces α-1,4, mientras que la segunda es una molécula de cadena más larga y ramificada, formada por cadenas de D-glucosa unidas por enlaces α-1,4 y ramificaciones α-1,6 cada 10-15 residuos.

En el endospermo, la síntesis del almidón ocurre en el amiloplasto, un plastidio sin aparato fotosintético dedicado exclusivamente a esta función (Fig. 4). En la síntesis del almidón, la glucosa-1 fosfato es transportada al amiloplasto y utilizada como sustrato por varias enzimas que actúan de forma secuencial formando la ruta de síntesis. La ADP glucosa pirofosforilasa, varias almidón-sintasas y enzimas ramificadas del almidón se encuentran entre estas enzimas. Las mismas han sido muy estudiadas en los últimos años debido a la importancia de la composición del almidón para muchas de sus propiedades tecnológicas.

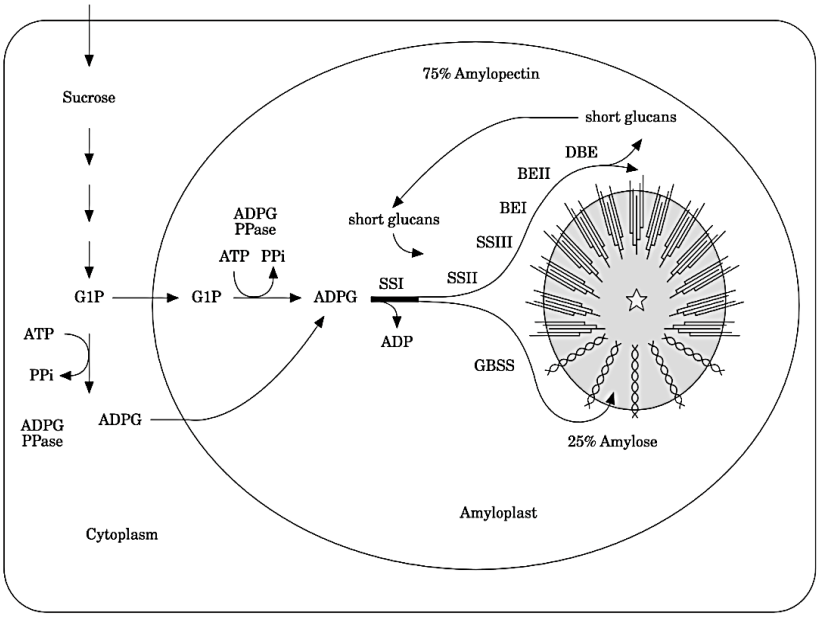


**Figura 4.** Síntesis del almidón en cereales (Rahman et al. 2000).

En el trigo se conocen tres tipos de almidón-sintasas que actúan en el endospermo:

- La almidón-sintasa unida al granulo I (*GBSSI, Granule Bound Starch Synthase* I) o proteína waxy.
- La almidón-sintasa I (SSI, *Starch Synthase* I o SGP1, *Starch Granule Protein* 1).
- La almidón-sintasa II (SSII, *Starch Synthase* II o SGP3, *Starch Granule Protein* 3).

La proteína waxy es la única responsable de la síntesis de la amilosa en el grano, mientras que el resto de sintasas están relacionadas con la síntesis de la amilopectina. Igualmente ha sido descrita una enzima ramificadora del almidón conocida como SBEII (*Starch Branching Enzyme* o SGP2, *Starch Granule Protein* 2) y una enzima desramificante (DBE, *Debranching Enzyme*), ambas involucradas también en la síntesis de la amilopectina.

Una de las proteínas más estudiadas involucrada en la síntesis del almidón es, sin duda, la proteína waxy. La misma está sintetizada por el gen *Wx,* situado en el cromosoma 7 de la mayoría de las especies de la familia *Poaceae* (Chao et al. 1989; Ainsworth et al. 1993). En el trigo harinero podemos encontrar tres proteínas waxy codificadas para los genes *Wx-A1* -cromosoma 7AS-, *Wx-B1* -cromosoma 4AL por una translocación de un segmento del cromosoma 7BS-, y *Wx-D1* -cromosoma 7DS- (Yamamori et al. 1994) con un peso molecular de 60,1, 59,2 y 59,0 kDa, respectivamente (Fujita et al. 1996). Se han encontrado alelos nulos para cada uno de los loci *waxy*; estos alelos nulos dan lugar a la ausencia de la proteína waxy correspondiente, resultando en una disminución en el contenido de amilosa (Nakamura et al. 1993a; Demeke et al. 1997; Graybosch 1998).

El almidón, como componente mayoritario del grano, tiene influencia en las propiedades funcionales de la harina de trigo para la fabricación de los diferentes productos. Sus componentes (amilosa y amilopectina) interactúan con el resto de los componentes del grano de trigo durante el amasado, influenciando enormemente la absorción de agua, los tiempos de fermentación requeridos por las masas, así como, el enranciamiento y las propiedades de textura del producto elaborado (Schoch y French 1947; Lee et al. 2001; Morita et al. 2002a; Van Hung et al. 2007). El almidón es además particularmente importante para determinar las propiedades de hinchamiento y formación de la pasta para la fabricación de los tallarines orientales (*noodles*), propiedades que son determinadas por la proporción amilosa/amilopectina; de hecho para estos usos se prefieren harinas de reducido contenido en amilosa (Peña 2002). Por otra parte, dado que se considera que estos almidones pueden ser utilizados en la elaboración de productos alimentarios refrigerados y congelados, debido a la baja tasa de retrogradación (insolubilización y precipitación espontánea del almidón, principalmente de las moléculas de amilosa, debido a la reorientación y unión por puentes de

hidrógeno de las cadenas lineales) de este tipo de almidón (Graybosch 1998). Además, una disminución en el contenido en amilosa contribuye a retardar el enranciaminto de los panes (Lee et al. 2001; Morita et al. 2002b). En este contexto, se buscó el modo de generar trigos con almidones waxy o libres de amilosa, mediante el cruzamiento entre un cultivar japonés (cv. Kanto107; *Wx-A1b*, *Wx-B1b*, *Wx-D1a*) y otro chino (cv. BaiHuo; *Wx-A1a, Wx-B1a, Wx-D1b*) que contenían alelos nulos para los diferentes genes *Wx* (Nakamura et al. 1993b).

**Los recursos fitogenéticos: la lucha contra la erosión genética**

Las plantas cultivadas han sufrido un proceso selectivo muy profundo, debido a la domesticación que las ha cambiado radicalmente con respecto a las formas silvestres de las que partían; esto además ha dado lugar a una gran variación genotípica por adaptación a distintos ambientes, manejos y usos. En el caso del trigo, esto produjo la generación de variedades locales con características agronómicas adaptadas a las zonas de cultivo, y con características de calidad asociadas a los diferentes usos de dichas zonas.

La evolución de la Agricultura durante el pasado siglo fue dando lugar al estrechamiento de la base genética de los cultivos en busca de variedades de alto rendimiento, hasta el punto de hacer prácticamente desaparecer o reducir a niveles puramente testimoniales el gran número de variedades locales generadas previamente durante siglos de agricultura tradicional (Esquinas-Alcazar 2005). Este proceso produjo una deriva muy importante de genes que no son directamente afectados por la selección, como lo son los que regulan los tres tipos de proteínas mencionados a lo largo de esta introducción: puroindolinas, proteínas de reserva y proteínas sintetizadoras del almidón. En consecuencia, una gran parte de la variabilidad genética existente para estos genes podría haber desaparecido de los campos de cultivos.

Una forma de recuperar parte de esta variabilidad genética pérdida podría ser utilizando los materiales almacenados en los bancos de germoplasma (Brown et al. 1989). Otra alternativa para incrementar la variabilidad genética de los cultivos actuales sería utilizar las especies silvestres emparentadas, que podrían portar variantes de estos genes todavía no incorporados en los cultivares modernos actualmente utilizados en la Agricultura. Por todo ello, la caracterización de los recursos fitogenéticos contenidos en los bancos de germoplasma, así como de las especies silvestres emparentadas con los cultivos, constituyen un paso clave para su posterior incorporación en materiales modernos, vía introgresión, con el objetivo de aumentar su variabilidad genética.

Actualmente, el cultivo del trigo en España, al igual que en otros países, depende de una serie de cultivares muy relacionados genéticamente. Hay, por tanto, una necesidad de disponer de otros materiales que permitan ampliar la base genética del cultivo y que además sean donantes potenciales de genes útiles.

Las variedades locales, los cultivares obsoletos y algunas subespecies de trigo, apenas ya cultivados, podrían constituir una fuente importante de variación para caracteres de gran interés económico como resistencia a enfermedades y a situaciones adversas medioambientales (temperaturas extremas, déficit hídrico, etc.), junto con caracteres relacionados con la calidad del trigo.

**Los trigos españoles abandonados o infrautilizados como recursos genéticos**

Como se ha mencionado, la variabilidad genética del trigo en el pasado podría haber sido mayor que la actual, parte de la cual se encontraría conservada en las accesiones almacenadas en los bancos de germoplasma (Nevo et al. 1988). Otra parte de esta variabilidad podría hallarse presente en

los materiales todavía conservados en manos de agricultores tradicionales, por lo que en los últimos años la conservación *in situ* también está siendo considerada (Skovmand et al. 2001). Este tipo de conservación se ve favorecida por el interés reciente en los cultivos antiguos y 'autóctonos' de cada zona, debido a que cada vez se le da mayor importancia a los alimentos producidos según técnicas tradicionales.

Las evidencias arqueológicas han demostrado que el trigo se ha cultivado en la Península Ibérica desde el quinto milenio a.C. (Buxó i Capdevilla et al. 1997). Los restos arqueológicos encontrados incluyen tanto trigos vestidos como desnudos. Estas denominaciones hacen referencia a que las glumas permanezcan o no adheridas al grano después de la trilla.

Aunque el término «vestido» genera cierta controversia, ya que incluye en el amplio sentido muchas especies dentro del complejo genético del trigo como especies en el género *Haynaldia*, *Aegilops* o *Agropyron,* entre otros. En un sentido estricto, la denominación de trigos vestidos se aplica a tres cultivos concretos: escaña (*T. monococcum* ssp *monococcum*), la escanda menor, povia o emmer (*T. turgidum* ssp. *dicoccum*) y la escanda mayor o espelta (*T. aestivum* ssp. *spelta*), que se cultivaron en España hace algún tiempo, pero que en la actualidad podrían ser catalogados como cultivos abandonados o infrautilizados. Otras especies de trigo que se cultivaron en España en el pasado son: trigo de Polonia (*T. turgidum* ssp. *polonicum* L. em. Thell.), trigo poulard o rivet (*T. turgidum* L. ssp. *turgidum*) y trigo cabezorro o club (*T. aestivum* L. ssp. c*ompactum* Host. em. Mackey). Este último, caracterizado por su espiga compacta y que se distribuía por todo el Viejo Mundo (Filatenko y Hammer 2014), prácticamente ha desaparecido de España, manteniendo una cierta importancia comercial en el área del noreste del Pacifico de los Estados Unidos, donde su harina se utiliza principalmente para la fabricación de galletas.

La variabilidad de trigo en España fue estudiada durante el siglo XIX, por los botánicos españoles Mariano Lagasca y Simón de Rojas Clemente, que en su obra inédita «Ceres Hispánica», recopilaron ejemplares de los trigos cultivados en aquella época (Téllez Molina y Peña 1952). En este herbario, el trigo harinero (*T. aestivum* L. ssp *aestivum*; syn *T. vulgare* Vill.) está representado por 466 accesiones agrupadas en 22 variedades botánicas: ocho del Grupo *mutica* (sin barbas) y 14 del Grupo *aristata* (con barbas), que fueron además clasificadas de acuerdo a tres rasgos morfológicos adicionales de la espiga y el grano: color de la gluma, pilosidad de glumas, y color del grano. Este estudio permite la comparación de la variabilidad morfológica detectada en el siglo XIX, con la del presente, observándose que la mayoría de las variedades botánicas compiladas por estos botánicos sólo se encuentran conservadas en bancos de germoplasma.

Actualmente, se ha producido un resurgir de los cultivos abandonados o infrautilizados. Desde el punto de vista de la mejora del trigo, son una potencial fuente de genes para la mejora del cultivo en la actualidad. Entre estos cultivos podemos incluir los denominados como trigos criollos, vestigios de los trigos harineros llevados por los españoles a Centroamérica y Sudamérica durante los siglos XVI al XVIII, que se mantienen en manos de pequeños agricultores tradicionales como es en el caso de México. Indudablemente, estos trigos derivan de las antiguas variedades españolas, y algunos de ellos son representantes de variedades botánicas que han desaparecido de los cultivares modernos (Téllez Molina y Peña 1952).

En este mismo sentido, cabría valorar la utilidad de variedades tradicionales españolas como las de Andalucía, gran parte de las cuales desaparecieron o fueron relegadas, al igual que en otras regiones de España, a raíz de la introducción de variedades modernas más rendidoras y adaptadas a la cosecha mecanizada desde la década los 60s del siglo pasado. Si bien esto supuso la desaparición de numerosas variedades tradicionales, algunas de

ellas todavía se cultivan en zonas marginales, principalmente en la zona norte de la provincia de Granada, asociadas a la fabricación de productos tradicionales.

Otras fuentes importantes de variación de los trigos harineros, si bien nunca fueron cultivados en España, comprendería especies como:

- Trigo macha [*T. aestivum* L. ssp. *macha* (Dekapr. & AM Menabde) Mackey] es un trigo vestido endémico de la zona del Cáucaso, descubierto en el oeste de Georgia en 1929. Dorofejev (1971) sugirió que esta especie sería la forma superviviente de la hibridación original de trigos hexaploides de raquis quebradizos, mientras que los espeltas iraníes y europeos son formas secundarias que surgieron de *T. aestivum* ssp. *aestivum*. No obstante, actualmente se considera que en realidad el trigo macha procede del espelta y no al revés.
- El trigo enano de la India [*T. aestivum* L. ssp. *sphaerococcum* (Percival) Mackey], se originó en la India y Pakistán (Hosono 1954). Esta subespecie se caracteriza por sus granos hemisféricos junto con su baja talla con cañas cortas y resistentes al encamado, espigas compactas de aristas cortas, floración temprana, y resistencia a la roya amarilla causada por *Puccinia striformis* (Percival 1921).

Estas variedades de trigos abandonados o infrautilizados a menudo albergan una cantidad significativa de la diversidad genética y podrían ser alternativas para la elaboración de pan y otros productos. Pero antes de volver a introducirlos para su uso directo o como fuentes de variación en los programas de mejoramiento de trigo, su caracterización para los caracteres de interés resulta fundamental y sumamente aconsejable.

**Objetivo**

El objetivo general de esta Tesis Doctoral ha sido estudiar la variabilidad de las gluteninas, proteínas waxy y puroindolinas en colecciones de trigos abandonadas o infrautilizadas, de cara a su valoración tanto como posibles recursos en la mejora genética del trigo moderno como cultivos *per se*.

# CAPÍTULO I

# CARACTERIZACIÓN DE LA DIVERSIDAD GENÉTICA DE PUROINDOLINAS EN VARIEDADES LOCALES DE TRIGOS MEXICANOS

**Publicado como:**

Ayala M, Guzmán C, Alvarez JB, Peña RJ (2013) Characterization of genetic diversity of puroindoline genes in Mexican wheat landraces. *Euphytica* 190: 53 - 63

**Resumen**

La dureza del grano es un factor importante para determinar el rendimiento molinero del trigo harinero. Influye en la cantidad de almidón dañado generado durante la molienda, y, por lo tanto, en el uso final de una variedad determinada. Se analizaron ciento dos líneas de quince variedades locales mexicanas de trigo para la dureza del grano y su control genético. Dieciséis líneas eran duras y ochenta y seis eran de textura suave. La textura de todas las líneas duras podría explicarse por una mutación en cualquiera de los genes *Pina-D1* o *Pinb-D1*. En seis líneas duras no había amplificación de *Pina-D1*, lo que sugiere que este gen se ha eliminado (alelo *Pina-D1b*). Las diez líneas duras restantes mostraron la presencia tanto de *Pina-D1* y *Pinb-D1*. La secuenciación de los genes *Pinb-D1* en estas líneas duras reveló la presencia de dos alelos diferentes (*Pinb-D1b* y *Pinb-D1e*). Los resultados corroboran la importancia de estas variedades locales mexicanas antiguas como potenciales fuentes de diversidad genética en cuanto a características de calidad para el desarrollo de variedades de trigo moderno con diferentes texturas de granos.

**Palabras clave:** Trigo harinero, Friabilina, Variabilidad genética, Textura del grano.

**Abstract**

Grain hardness is a major factor determining the milling performance of common wheat. It determines the amount of damaged starch generated during milling, and therefore the end use of a given variety. One hundred and two lines from 15 Mexican wheat landraces were analyzed for grain hardness and for its genetic control. Sixteen lines were hard and 86 were soft-textured. All hard lines could be explained by a mutation in either the *Pina-D1* or *Pinb-D1* genes. In six hard lines there was no amplification of *Pina-D1*, suggesting that this gene was deleted (*Pina-D1b* allele). The remaining ten hard lines showed the presence of both *Pina-D1* and *Pinb-D1*. Sequencing the *Pinb-D1* genes of the hard lines revealed the presence of two different alleles (*Pinb-D1b* and *Pinb-D1e*). The results substantiate the importance of very old Mexican landraces as potential sources of genetic diversity for key quality traits in the development of modern wheat cultivars with different grain textures.

**Keywords:** Common wheat, Friabilin, Genetic variability, Grain texture.

**Introduction**

Grain hardness is an important quality parameter determining market classification and end use properties of common wheat (*Triticum aestivum* L. ssp. *aestivum*). Based on this trait, common wheat is classified into soft and hard (Pomeranz and Williams 1990). During flour milling or whole meal grinding soft wheat kernels fracture easily, producing fine-textured flours with low levels of damaged starch granules, which make this type of flour suitable for cookies, cakes, pastries, and some types of noodles (Morris and Rose 1996). Hard wheats produce coarser-textured flours that exhibit fracture planes and considerable amounts of broken starch granules. The high levels of starch damage in hard wheat flour allow high water absorption, making hard wheat highly suited for the manufacture of bread and other foods prepared with viscoelastic dough (Morris and Rose 1996).

The molecular-genetic basis of wheat grain hardness is now well established, being related with "friabilin", an Mr 15-kDa protein associated with the starch membrane and abundant in soft wheat starch, scarce in hard wheat starch, and absent in durum wheat (Greenwell and Schofield 1986). The main component of "friabilin" are puroindolines *a* and *b* (PINA and PINB); and a third minor protein called grain softness protein (GSP). These proteins are encoded by the *Pina-D1*, *Pinb-D1* and *Gsp* genes located in the *Ha* (*Hardness*) locus, which is present in the short arm of the chromosome 5D of common wheat (Morris 2002). It is a complex locus formed by ten closely linked genes (Li et al. 2008), but only two (*Pina-D1* and *Pinb-D1*) have been associated with grain hardness (Gautier et al. 1994; Rahman et al. 1994; Giroux and Morris 1997, 1998; Xia et al. 2008).

Although the use of species related to wheat has increased the numbers of alleles for both genes that confer soft endosperm (Morris and Bhave

2008), this grain texture in common wheat has been associated with the presence of the wildtype alleles (*Pina-D1a* and *Pinb-D1a*), whereas hard texture results from single or double mutations in both genes (reviewed in Morris 2002). These are either *null* mutations resulting in absence of transcription, or mutations causing amino acid substitutions in PINA or PINB. The mutations appear to have occurred independently and some can be tracked back to specific geographic areas (Chen et al. 2006, 2007b; Lillemo and Moris 2000; Lillemo et al. 2006; Moris et al. 2001; Tanaka et al. 2008).

It has been shown that not all the *Pin* mutations cause the same effect on grain hardness. For example, *Pina-D1b* tends to give harder endosperm than *Pinb-D1b* (Cane et al. 2004; Giroux et al. 2000; Martin et al. 2001). Although the known hardness mutations all confer large and somewhat similar changes in endosperm texture relative to soft wheat, the discovery of new alleles could broaden the genetic background for grain hardness and provide industry with grains more suitable for a range of uses. For this reason the screening of common wheat germplasm collections and related species to find new *Pin* alleles has been important (Chen et al. 2006; 2007a, b; Ikeda et al. 2005; Massa et al. 2004; Pickering and Bhave 2007; Ram et al. 2002).

The Mexican Creole wheats (landraces) are the last trace of common wheat carried by the Spanish to America during the period between the 16th and the 18th centuries. These wheats derive from old Spanish cultivars and landraces and their cultivation survives on small-scale traditional farms. Some of them are representatives of botanical varieties that are not included among modern cultivars, but they can still be identified in the unpublished herbarium '*Ceres Hispanica*', developed by Mariano Lagasca and Simon de Rojas Clemente during the Nineteenth Century (Téllez Molina and Peña 1952). A wide collection of these old wheats collected throughout Mexico

and kept in the Germplasm Bank of the International Maize and Wheat Improvement Center (CIMMYT) was evaluated for morphological traits and endosperm storage proteins (Caballero et al. 2010). Up to seven botanical varieties were detected among these lines; some of them have disappeared in modern wheat. In addition, five novel allelic variants for the *Glu-1* loci were detected, exemplifying the clear risk of genetic erosion of genes that occur at low frequencies. This type of variability makes these materials useful for evaluating other traits with key roles in determination of grain quality such as starch characteristics or grain hardness.

The aim of the current study was to evaluate the puroindoline composition of a collection of Mexican Creole wheat landraces and its relationship with grain hardness.

## Materials and methods

*Plant materials*

One hundred and two lines selected from 15 Mexican landraces collected from several Mexican states (Fig. 1) were obtained from the Germplasm Bank of the CIMMYT (Supplementary Table 1). These lines were multiplied at the CIFA-IFAPA Experimental station at Córdoba, Spain, during 2007-08 in 1 m row plots without replication, but with standard agronomic practices for the region (175 kg/ha of N, 90 kg/ha of P and 90 kg/ha of K). Common wheat cv. Chinese Spring and durum wheat cv. Langdon were included as standards.

*Evaluation of quality traits*

Mean kernel weight was determined on 20 g samples with broken grains removed. Test weight (kg/Hl) was measured using a 250-ml chondrometer. Grains from each line were milled using a cyclone mill (Foss 1093 Cyclotec sample mill) fitted with a 1 mm sieve. Protein content was

determined by the Kjeldahl method (%$N$ × 5.7, dry matter). Grain hardness was measured by particle size index (PSI) as described by Williams et al. (1988). Five grams of flour were sifted through a 200 mesh (75 $\mu$m) over 5 min. The PSI value was calculated as the percentage of sifted flour.


**Figure 1.** Geographical distribution of the Mexican creole wheat accessions evaluated in the current study. The *initial* indicated the Mexican states were each landrace was collected: *BCS* Baja California Sur, *CHIH* Chihuahua; *COAH* Coahuila; *GTO* Guanajuato; *HGO* Hidalgo; *MEX* Estado de Mexico; *MICH* Michoacan; *NL* Nuevo Leon, *OAX* Oaxaca, *PBL* Puebla, *PUE* Puebla, *QRO* Queretaro and *TXL* Tlaxcala. The numbers are the last two numbers of the year they were collected.

*DNA extraction and PCR amplification of the* Pina-D1 *and* Pinb-D1 *genes*

Genomic DNA was extracted from young leaves of single plants by the CTAB method (Stacey and Isaac 1994). The *Pina-D1* gene was amplified using primers designed by Lillemo et al. (2006): forward, 5'-CATCTATTCATCTCCACCTGC-3', and reverse, 5'-GTGACAGTTTATTAGCTAGTC-3'; whereas primers designed by Gautier et al. (1994) were used for amplifying *Pinb-D1*: forward, 5'-ATGAAGACCTTATTCCTCCTA-3', and reverse, 5'-

TCACCAGTAATAGCCACTAGGGAA-3'). Each 15 $\mu$l reaction included 50 ng DNA, 1 or 1.5 mM $MgCl_2$ (for *Pina-D1* and *Pinb-D1*, respectively), 0.2 $\mu$M of each primer, 0.2 mM dNTPs, 1.5 $\mu$l 10× PCR buffer and 0.75U DNA polymerase (Promega). The PCR conditions included an initial denaturation step of 3 min at 94°C followed by 35 cycles as follows: 45 s at 94°C, 1 min 30 s at 60°C, then 45 s at 72°C. After the 35 cycles there was a final extension of 5 min at 72°C. Due to the similar size of the PCR products for each gene, these were restricted by endonucleases whose effectiveness for detecting mimetic alleles have been validated (Pickering and Bhave 2007; Wang et al. 2008; Guzmán et al. 2012).

The presence of alleles *Pina-D1r* and *Pina-D1s* was determined using the protocol described by Ikeda et al. (2010).

Amplification products were fractionated in vertical PAGE gels in discontinuous Tris-HCl buffer system (pH 6.8/8.8) with 8% (w/v, C 1.28%) polyacrylamide concentration, whereas the digested fragments were analysed in PAGE gels with 10% polyacrylamide concentration (C 3.0%). In both case, Tris/glycine buffer was used and the bands were visualized by ethidium bromide staining and photographed under ultraviolet light using Kodak Digital ID software ver. 3.6.

The PCR products were purified with SureClean product insert (Bioline), and a small sample was cloned in the pGEMT-easy vector (Promega). Three positive clones were sequenced in both directions with M13 universal primers on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). The sequences obtained were compared with the available puroindoline sequences in NCBI database (http://www.ncbi.nlm.nih.gov) using the Geneious Pro software ver. 5.0.3 (Biomatters Ltd.).

*Statistical analysis*

The relationship between puroindoline composition and PSI values was statistically analysed by *t*-Student tests.

Genetic variability in each landrace was assessed using the number of alleles per locus (*A*), the effective number of alleles per locus (*Ne*), and the mean gene diversity (*He*) according to the unbiased estimate method of Nei (1972, 1973). Genetic diversities were estimated using Nei's diversity index, *He* (Nei 1972, 1973). The genetic diversity over all landraces (*Ht*) together with the average genetic diversities within (*Hs*) and among (*Dst*) landraces were calculated according to Nei (1973). The relative magnitude of genetic differentiation among landraces, *Gst*, is estimated as *Dst*/*Ht*. The Ewens-Watterson test for neutrality (1000 permutations) was performed for each locus to detect the possible effects of selection according to the algorithm of Manly (1985). Ohta's two-locus population subdivision analysis was used to test the linkage disequilibrium between both loci (Ohta 1982a, b), and to measure the components of variance of the disequilibrium: $D_{IT}^2$: total variance of disequilibrium; $D_{IS}^2$: variance of within group disequilibrium; $D_{ST}^2$: variance of correlation of genes of the two loci of different gametes of one group relative to that of the total population; $D'_{IS}^2$: variance of the correlation of genes of the two loci of one gamete in a group relative to that of the total population; and $D'_{ST}^2$: variance of the disequilibrium of the total population. Popgene software version 1.3.2 (Yeh et al. 1997) was used for estimation of the above parameters.

## Results

*Variation in grain hardness*

All the lines showed well-formed plump seeds, with similar medium grain size, and high (mean 15.2%) protein content (data not shown). Regarding grain hardness, the population showed a mean PSI of 45.8 ± 7.5%,

ranging between 24 and 58% (Fig. 2). Cv. Chinese Spring (*Pina-D1a/Pinb-D1a*) used as reference for soft wheat (Giroux and Morris 1997), showed a PSI value of 52%. More than 75% of the lines showed soft to hard texture ranging between 42 and 54%. Four lines were very soft with high PSI values (56-58%) and 20 lines were very hard with values under 40%. However, no line showed a value less than that shown by cv. Langdon (PSI, 20%) used as reference for durum grain hardness.

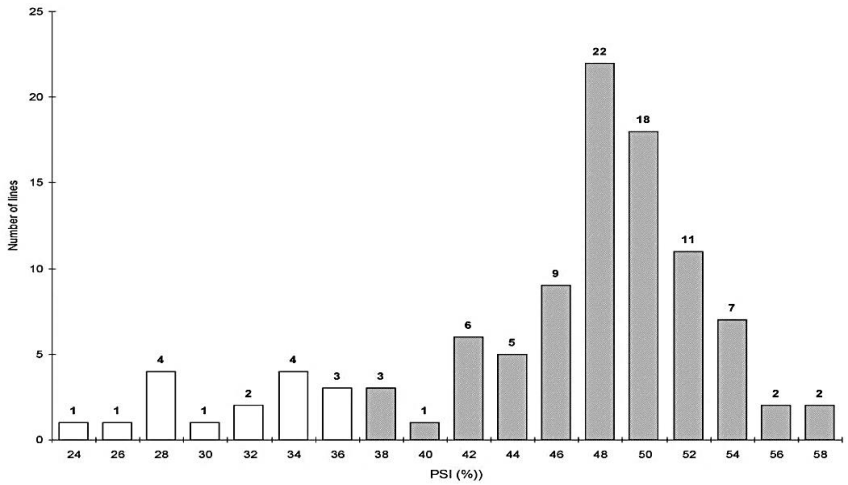


**Figure 2.** PSI frequency distribution of the lines evaluated. *White bars* indicates hard lines, *grey bars* corresponds to soft lines.

*PCR analysis of the* Pina-D1 *and* Pinb-D1 *genes*

According to Morris and Bhave (2008) soft genotypes in common wheat are associated with the presence of wildtype alleles for both *Pin* genes, whereas single or double mutant alleles at one or both loci generate hard genotypes. Consequently, the lines were analysed for the presence of *Pin* alleles by selective PCR amplification of the wildtype alleles using genomic DNA. All lines with PSI values ≤ 40% (20 lines), together with a random wide sample lines spanning all classes (9 lines), were analysed by PCR amplification (Fig. 2). For the *Pina-D1* gene, most of the lines presented a

PCR product with size around 524 bp (Fig. 3a), except for six lines (CWI60059, CWI60579, CWI62903, CWI62911, CWI65378 and CWI65379) that generated no amplification product (Fig. 3a, lanes 2, 3, 4 and 9). In order to exclude the possibility that the absence of amplification was due to changes in the annealing sequence of the primers, PCR amplification of *Pina-D1* was carried out using another pair of primers (Gautier et al. 1994). The absence of *Pina-D1* amplicons also with these primers (data not shown) confirmed that these lines did not contain a sequence corresponding to the *Pina-D1* gene. The possible presence of other rare *null* alleles for the *Pina-D1* gene (*Pina-D1r* and *Pina-D1s*) was tested using primers designed by Ikeda et al. (2010). In both cases, no amplicons were produced, and consequently these materials were considered PINA *null* (tentatively *Pina-D1b* allele). These lines were considered hard, with a PSI mean = 30.0 ± 4.2% (Table 1). When the rest of the lines were evaluated by restriction enzymes, only one sequence type was identified.

**Table 1.** Allelic composition for puroindolines genes and PSI values of the lines evaluated

| Puroindoline alleles | N | PSI (% ± s.d.) | Range | Phenotype[a] |
|---|---|---|---|---|
| *Pina-D1a/Pinb-D1a* | 86 | 48.5 ± 4.2 | 38.0 - 58.0 | Soft |
| *Pina-D1a/Pinb-D1b* | 2 | 32.0 ± 5.7 | 28.0 - 36.0 | Hard |
| *Pina-D1a/Pinb-D1e* | 8 | 32.0 ± 3.5 | 28.0 - 36.0 | Hard |
| *Pina-D1b/Pinb-D1a* | 6 | 30.0 ± 4.2 | 24.0 - 34.0 | Hard |

*s.d.* Standard deviation
[a]According to Morris and Bhave (2008)

All lines showed the expected amplification product for the *Pinb-D1* gene, including the six lines without a *Pina-D1* gene (Fig. 3b). Unfortunately, the PCR products showed little variation for mobility, and only one line showed a band with minor mobility (Fig. 3b, lane 11). However, the use of

the endonucleases permitted detection of further variation (Wang et al. 2008). With two of the endonucleases (*BstXI* and *BsrBI*) differences were detected among the amplicons (Fig. 4). *BstXI* cuts in the target sequence CCANNNNNNTGG. Eight lines (CWI60507, CWI60509, CWI60541, CWI63279, CWI64800, CWI65354, CWI65420 and CWI65483) showed the loss of this restriction site suggesting that these lines could have the *Pinb-D1e* allele (Fig. 4a, lanes 4, 5, 6 and 7). In addition, digestion with *BsrB1* (GAGCGG) showed a different banding pattern: two lines (CWI60005 and CWI60316) showed two restriction sites instead of one; a variation associated with the *Pinb-D1b* allele (Fig. 4b, lane 11), that generates an amplicon with slightly higher mobility (Fig. 3b, lane 11). Both mutations produce amino acid changes (Gly-46 to Ser-46 for *Pinb-D1b*, and Trp-39 to stop codon for *Pin-D1e*) and generate hard genotypes. The former is a frequent allele (Giroux and Morris 1997), which generates a PINB protein that is not completely functional, whereas the rare *Pinb-D1e* allele (Morris et al. 2001) is PINB null. In the present study the lines that contained these putative alleles showed PSI values of 32.0 ± 5.7% and 32.0 ± 3.5%, respectively (Table 1).

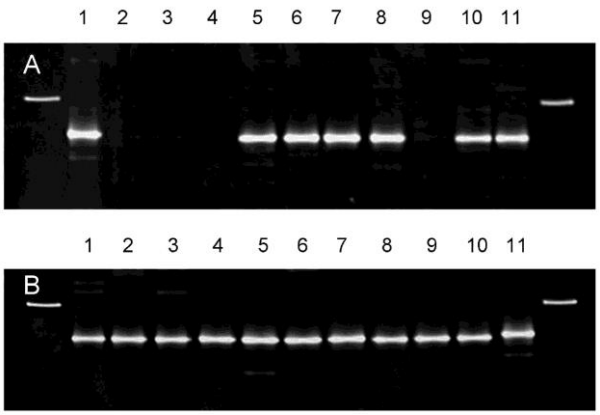


**Figure 3. a** Pina amplification. **b** Pinb amplification. *Lanes* are as follows: *1* line CWI76845; *2* line CWI60579; *3* line CWI62903; *4* line CWI62911; *5* line CWI60507; *6* line CWI60509; *7* line CWI60541; *8* line CWI63279; *9* line CWI60059, *10* cv. Chinese Spring, and *11* line CWI60316.

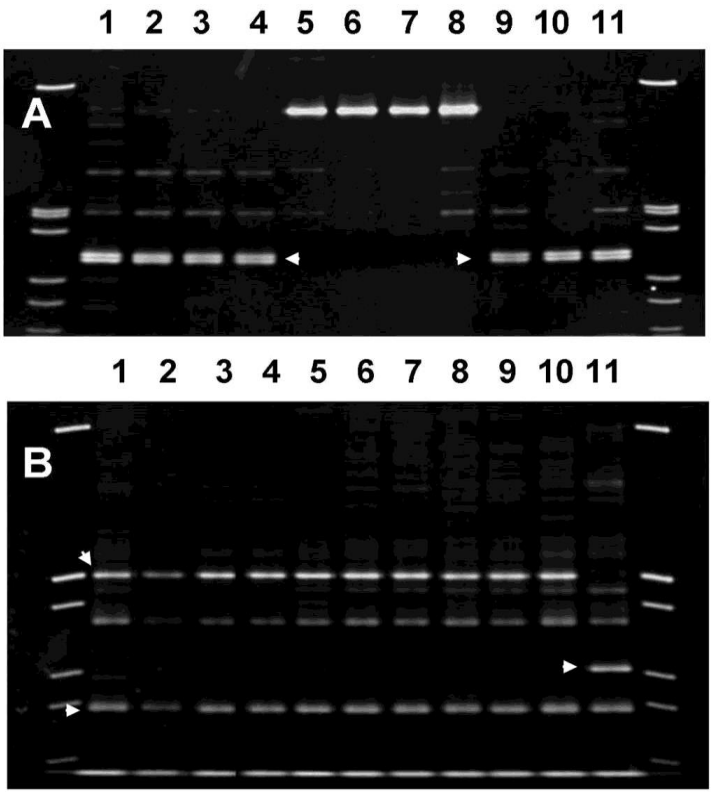


**Figure 4.** *Pinb-D1* sequences digested with *BstXI* (**a**) and *BsrBI* (**b**). Digested fragments are indicated by arrowheads. Line order is the same as in Fig 3.

The restriction pattern indicates the presence of a single nucleotide polymorphism (SNP) in the target sequence, and only the complete sequence of the amplicon can univocally characterize the correct allele. Consequently, all the amplicons were sequenced and compared with the wildtype allele sequences (*Pina-D1a* and *Pinb-D1a*) available in the NCBI database. In the lines with PSI values above 38%, all the sequences were the same as *Pina-D1a* or *Pinb-D1a*. In the PINA null (*Pina-D1b*) lines no change was detected in the *Pinb-D1* sequence and therefore these were classified as *Pinb-D1a*. The putative alleles (*Pinb-D1b* and *Pinb-D1e*) present in the remaining hard lines were confirmed by sequence (Fig. 5). Both sequences are available in the NCBI database (*Pinb-D1b*: JN626223; *Pinb-D1e*: JN626222).

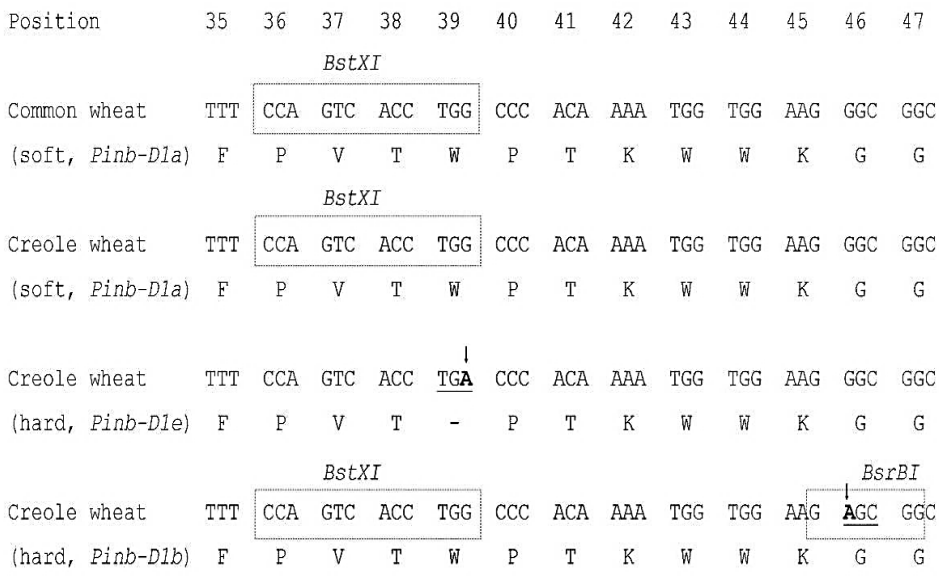


**Figure 5.** DNA *Pinb-D1* sequence and deduced amino acid sequence of the region where both mutations found were present. Codons of the mutations are *underlined* and mutated bases are in *bold*. Target sequences of restriction enzymes are indicated by *boxes*.

Although these data must be considered preliminary due to the different backgrounds of the landraces, the comparisons between the wildtype genotype (*Pina-D1a/Pinb-D1a*) and the group of landraces with *Pina-D1b, Pinb-D1b* or *Pinb-D1e* alleles showed that these latter alleles have similar effect on PSI and were significantly different from wildtype (Table 2).

**Table 2.** Mean differences in PSI values associated with the variation in puroindoline genes.

| Puroindolines composition (*Pina-D1/Pinb-D1*) | | *N* | | PSI (% ± s.d.) | | |
|---|---|---|---|---|---|---|
| x | y | $x$ | $y$ | $x$ | $y$ | t-Student test |
| *a/a* | *a/b* | 86 | 2 | 48.5 ± 4.2 | 32.0 ± 5.6 | 5.48*** |
| *a/a* | *a/e* | 86 | 8 | 48.5 ± 4.2 | 32.0 ± 3.5 | 10.77*** |
| *a/a* | *b/a* | 86 | 6 | 48.5 ± 4.2 | 30.0 ± 4.2 | 10.45*** |

*s.d.* Standard deviation.
*** Significant at $P = 0.001$.

*Genetic structure*

The 102 lines were grouped according to their puroindoline haplotypes and the site from which they were collected (Table 3). Data showed that the *A* values were independent of the number of accessions of each landrace. Only five of 15 landraces showed genetic diversity; the rest showed no variation (*He* = 0.000). In the landraces that were variable the difference observed between the *A* and *Ne* values indicated that the variation was in danger of genetic erosion ranging from 13.3% for PUE94 and 42.0% for HGO94 (Erosion percentage = [1 – *A*/*Ne*] x 100). Across all landraces this danger is clear where the value is 53.0%. Hard haplotypes (16 accessions) were detected in five landraces; four of them were from central Mexican states (Guanajuato, Hidalgo, Mexico and Puebla) and the fifth was from northern Mexico (Chihuahua State). HGO94 was the more diverse with all haplotypes, although its *He* value (0.293) was low due to the distribution of these haplotypes were the wildtype (*Pina.D1a*/*Pinb-D1a*) was clearly hegemonic. The highest *He* value (0.370) was detected in CHICH95 that had two of the three hard haplotypes.

Additional characterization of the diversity at the *Pina-D1* and *Pinb-D1* loci is presented in Table 4. The average total genetic diversity (*Ht*) across both loci in the 15 landraces was 0.091, with the *Ht* value for *Pina-D1* being approximately half of the *Pinb-D1* (0.057 vs 0.125). This diversity can be divided in two components, *Hs* and *Dst* (Table 4), measuring the genetic diversity within and among landraces (Nei 1973). The average relative differentiation among landraces was *Gst* = 16.5% (ranging from 12.3% for *Pina-D1* to 19.2% for *Pinb-D1*), indicating that most of the genetic diversity is within landraces.

**Table 3.** Genetic diversity and distribution of puroindoline haplotypes in the Mexican Creole wheat lines evaluated according to their geographical origin.

| | | Genetic diversity | | | Haplotypes *Pina-D1/Pinb-D1* | | | |
|---|---|---|---|---|---|---|---|---|
| Landrace | N | *A* | *Ne* | *He* | *a/a* | *a/b* | *a/e* | *b/a* |
| BCS95 | 5 | 1.00 | 1.00 | 0.000 | 5 | - | - | - |
| CHIH95 | 10 | 2.00 | 1.60 | 0.370 | 5 | - | 3 | 2 |
| CHIH96 | 4 | 1.00 | 1.00 | 0.000 | 4 | - | - | - |
| COAH90 | 5 | 1.00 | 1.00 | 0.000 | 5 | - | - | - |
| GTO95 | 5 | 1.50 | 1.24 | 0.160 | 4 | - | 1 | - |
| HGO94 | 20 | 2.50 | 1.45 | 0.293 | 13 | 2 | 3 | 2 |
| MEX92 | 4 | 1.00 | 1.00 | 0.000 | 4 | - | - | - |
| MEX94 | 14 | 1.50 | 1.16 | 0.122 | 12 | - | - | 2 |
| MICH89 | 4 | 1.00 | 1.00 | 0.000 | 4 | - | - | - |
| NL90 | 3 | 1.00 | 1.00 | 0.000 | 3 | - | - | - |
| OAX93 | 14 | 1.00 | 1.00 | 0.000 | 14 | - | - | - |
| PBL94 | 2 | 1.00 | 1.00 | 0.000 | 2 | - | - | - |
| PUE94 | 4 | 1.50 | 1.30 | 0.188 | 3 | - | 1 | - |
| QRO94 | 7 | 1.00 | 1.00 | 0.000 | 7 | - | - | - |
| TXL92 | 1 | 1.00 | 1.00 | 0.000 | 1 | - | - | - |
| **Overall landrace** | 102 | 2.50 | 1.17 | 0.145 | 86 | 2 | 8 | 6 |

*BCS*: Baja California Sur; *CHIH*: Chihuahua; *COAH*: Coahuila; *GTO*: Guanajuato; *HGO*: Hidalgo; *MEX*: Estado de México; *MICH*: Michoacan; *NL*: Nuevo León; *OAX*: Oaxaca; *PBL*: Puebla; *PUE*: Puebla; *QRO*: Queretaro; and *TXL*: Tlaxcala. The numbers are the last two numbers of the year they were collected.
*A* mean number of allele per locus, *Ne* mean effective number of allele, *He* expected heterozygosity.

The Ewens-Watterson test showed that the allelic frequencies for both loci were selectively neutral (Table 5). Linkage disequilibrium evaluated according to the method of Ohta (1982a, b) established that each landrace behaved as a subpopulation (Table 5). Because of the inbreeding nature of these cultivated materials, migration is limited and highly correlated with seed exchange among farmers. Data showed that the variance of the expected

allelic associations among landraces ($D'_{IS}{}^2$) was larger than $D'_{ST}{}^2$ (variance of the disequilibrium of the total population) and that $D_{ST}{}^2$ (variance of correlation of genes of the two loci of different gametes of one subpopulation relative to that of the total population) was also larger than $D_{IS}{}^2$ (variance of within subpopulation disequilibrium), suggesting that the observed linkage disequilibrium is mainly a consequence of random genetic drift.

**Table 4.** Differentiation of the normalised diversity of puroindoline composition within and among 15 landraces of Mexican Creole wheat.

| Locus | Nº alleles | *Ht* | *Hs* | *Dst* | *Gst* (%) |
|---|---|---|---|---|---|
| *Pina-D1* | 2 | 0.057 | 0.050 | 0.007 | 12.3 |
| *Pinb-D1* | 3 | 0.125 | 0.101 | 0.024 | 19.2 |
| Total | | 0.091 | 0.076 | 0.015 | 16.5 |

*Ht* total gene diversity, *Hs* average gene diversity within landraces, *Dst* average gene diversity between landraces, *Gst* gene diversity between landrace, relative to *Ht*.

**Table 5.** Ewens-Watterson test for neutrality and Ohta's two-locus analysis of population subdivision for the 15 landraces of Mexican Creole wheats evaluated.

| Ewens-Watterson test | | | | | |
|---|---|---|---|---|---|
| Locus | *k* | Obs. *F* | Mean ± s.e.[a] | $L95^2$ | $U95^2$ |
| *Pina* | 2 | 0.889 | 0.807 ± 0.029 | 0.503 | 0.981 |
| *Pinb* | 3 | 0.820 | 0.663 ± 0.034 | 0.355 | 0.961 |
| Ohta's analysis[b] | | | | | |
| Locus A – Locus B | $D_{IT}{}^2$ | $D_{IS}{}^2$ | $D_{ST}{}^2$ | $D'_{IS}{}^2$ | $D'_{ST}{}^2$ |
| *Pina-D1 – Pinb-D1* | 0.043 | 0.001 | 0.037 | 0.039 | 0.004 |

*k* number of alleles, *F* homozygosity, *s.e.* standard error

[a] Calculated using 1,000 simulated samples

[b] For paramenters od the Ohta`s analysis see "Materials and methods" section

**Discussion**

In this survey, a collection of Mexican landraces was analyzed for grain texture and characterized at the molecular level by puroindoline composition. Most of the lines were soft, indicating that both puroindoline genes were functional. However, 16 selections were classified as hard, and were explained by mutations in either *Pina-D1* or *Pinb-D1*, in agreement with results published earlier (Chen et al. 2006; 2007a,b; Giroux and Morris 1997; 1998; Ikeda et al. 2005). In six of the 16 hard lines there was no amplification of *Pina-D1*, suggesting that this gene was deleted in these lines (*Pina-D1b* allele) as reported by Giroux and Morris (1998). Although this allele is present at high frequency in CIMMYT wheat germplasm (Lillemo et al. 2006) as well as in Indian and Chinese cultivars (Chen et al. 2007a, b; Ram et al. 2002) the current study showed that Mexican landraces represent a new source for this null allele.

The remaining ten hard lines possessed both the *Pina-D1* and *Pinb-D1* genes. Sequencing the *Pin* genes in these lines revealed the presence of two different mutations in the *Pinb-D1* gene compared to the wildtype allele carried by soft lines. Two lines possessed the *Pinb-D1b* allele (Gly-46 to Ser-46), a common allele that is the primary source of grain hardness worldwide. The other hard lines had rare mutation described by Morris et al. (2001) and named *Pinb-D1e*. This mutation has a premature stop codon at position 39 of the mature protein. This allele was later found in Chinese cultivars (Chen et al. 2007a, b; Wang et al. 2008). The eight Mexican landraces lines of that carry this allele could be used to widen the genetic variability of *Pin loci* in modern wheats, which has been narrowed down as has been shown by Lillemo et al. (2006) who only found two different hardness alleles (*Pina-D1b*, *Pinb-D1b*) in a representative sample of 373 bread wheat from the breeding program at CIMMYT. These landraces, not frequently utilized in modern

breeding programmes, increase the number of lines having a particular hardness allele.

Therefore, breeders could select among more lines for other desirables traits and then use the line with best combination of traits as a parent. In fact, some of these landraces have been already used at CIMMYT as, sources if traits such as drought and heat tolerance and for increasing iron and zinc grain concentrations in the grain (Velu et al. 2011). Therefore, the information provided in the current study will be useful for the breeders.

Some authors have suggested that *Pina-D1b* genotypes are harder than *Pinb-D1b* genotypes (Giroux et al. 2000; Martin et al. 2001), but our data did not show differences in grain hardness between mutants of the *Pina-D1* and *Pinb-D1* genes (Table 1), although the lowest PSI values (24 and 26%) were found in two lines with the *Pina-D1b* allele. However, grain hardness may be affected by environment and the because the genetic background of these materials is very different from those examined in other studies more precise measurements need to be made in order to assess the true effect of each allele on grain hardness. In this respect, the use of NILs or RILs derived from crosses involving the current materials should be considered.

Several studies have been carried out on the distribution of the puroindoline haplotypes around the world (Cane et al. 2004; Chen et al. 2006; Giroux and Morris 1998; Ikeda et al. 2005; Lillemo et al. 2006; Morris et al. 2001; Pickering and Bhave 2007; Ram et al. 2002; Tanaka et al. 2008). The Mexican landraces evaluated in this study show the characteristics of ancestral wheat varieties introduced during European colonization (Tanaka et al. 2008), and are the last representatives of the wheat taken by the Spanish to Mexico. The disequilibrium linkage analysis suggests that the distribution of the rare alleles is mainly a consequence of genetic drift. Current data suggest that probably these rare alleles arrived in Hidalgo state and were distributed to neighbouring states, and subsequently taken to the north to Chihuahua

state. This hypothesis is in consonance with previous data from analysis of morphological traits and HMWG composition in the same collection (Caballero et al. 2010). In the previous study, the CHIH95 and HGO94 landraces showed only a small difference in Nei's distance. Also, the landraces with some of the non-wildtype haplotypes (a/b, a/e or b/a) appeared to group with these two landraces (Caballero et al. 2010). On the other hand, because of the origin of these wheats, the presence of the rare puroindoline haplotypes may be explored in old local varieties of Spanish common wheat.

In conclusion, although this collection of Mexican landraces has no novel alleles of the puroindoline genes, they include one rare allelic variant (*Pinb-D1e*) that could influence grain hardness. Up to now, that allele has been found in only seven lines of bread wheat (Chen et al. 2007b; Edwards et al. 2010; Morris et al. 2001). Other work has successfully used these materials as sources of a range of agronomic and nutritional traits. The results therefore provide evidence of the importance of these genetic materials as sources of genetic diversity for use in breeding programs.

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## Supplementary

**Supplementary Table 1:** Origin and puroindoline alleles for the all accessions of Mexican Creole wheat landraces

| Accession | Landrace | *Pina-D1* | *Pinb-D1* |
|---|---|---|---|
| CWI35248 | NL90 | a | a |
| CWI35276 | NL90 | a | a |
| CWI35289 | NL90 | a | a |
| CWI59740 | PLBL94 | a | a |
| CWI59797 | PLBL94 | a | a |
| CWI59894 | QRO94 | a | a |
| CWI59988 | QRO94 | a | a |
| CWI60064 | HGO94 | a | a |
| CWI60316 | HGO94 | a | b |
| CWI60334 | HGO94 | a | a |
| CWI60426 | HGO94 | a | a |
| CWI60434 | HGO94 | a | a |
| CWI60507 | HGO94 | a | e |
| CWI60509 | HGO94 | a | e |
| CWI60527 | HGO94 | a | a |
| CWI60529 | HGO94 | a | a |
| CWI60541 | HGO94 | a | e |
| CWI60550 | HGO94 | a | a |
| CWI60556 | HGO94 | a | a |
| CWI60558 | HGO94 | a | a |
| CWI60579 | HGO94 | b | a |
| CWI60598 | HGO94 | a | a |
| CWI60613 | HGO94 | a | a |
| CWI61125 | MEX94 | a | a |
| CWI61127 | MEX94 | a | a |
| CWI61155 | MEX94 | a | a |
| CWI61168 | MEX94 | a | a |
| CWI61190 | MEX94 | a | a |
| CWI62321 | MEX94 | a | a |
| CWI62773 | MEX94 | a | a |
| CWI62790 | MEX94 | a | a |
| CWI62877 | MEX94 | a | a |
| CWI62903 | MEX94 | b | a |
| CWI62911 | MEX94 | b | a |
| CWI63279 | PUE94 | a | e |
| CWI65231 | CHIH95 | a | a |
| CWI65249 | CHIH95 | a | a |

| Accession | Landrace | *Pina-D1* | *Pinb-D1* |
|---|---|---|---|
| CWI65297 | CHIH95 | a | a |
| CWI65300 | CHIH95 | a | a |
| CWI65354 | CHIH95 | a | e |
| CWI65378 | CHIH95 | b | a |
| CWI65379 | CHIH95 | b | a |
| CWI65420 | CHIH95 | a | e |
| CWI65483 | CHIH95 | a | e |
| CWI76852 | OAX93 | a | a |
| CWI76873 | OAX93 | a | a |
| CWI76894 | OAX93 | a | a |
| CWI76896 | OAX93 | a | a |
| CWI76899 | OAX93 | a | a |
| CWI76904 | OAX93 | a | a |
| CWI76905 | OAX93 | a | a |
| CWI76907 | OAX93 | a | a |
| CWI76922 | OAX93 | a | a |
| CWI31298 | MICH89 | a | a |
| CWI31412 | MICH89 | a | a |
| CWI31482 | MICH89 | a | a |
| CWI31567 | MICH89 | a | a |
| CWI33379 | COAH90 | a | a |
| CWI33413 | COAH90 | a | a |
| CWI33422 | COAH90 | a | a |
| CWI33434 | COAH90 | a | a |
| CWI34294 | COAH90 | a | a |
| CWI49661 | MEX92 | a | a |
| CWI49714 | MEX92 | a | a |
| CWI49735 | MEX92 | a | a |
| CWI49747 | MEX92 | a | a |
| CWI49943 | TXL92 | a | a |
| CWI53661 | PUE94 | a | a |
| CWI53666 | PUE94 | a | a |
| CWI53673 | PUE94 | a | a |
| CWI59884 | QRO94 | a | a |
| CWI59939 | QRO94 | a | a |
| CWI59977 | QRO94 | a | a |
| CWI59993 | QRO94 | a | a |
| CWI60001 | QRO94 | a | a |
| CWI60005 | HGO94 | a | b |
| CWI60059 | HGO94 | a | a |
| CWI60114 | HGO94 | a | a |
| CWI60139 | HGO94 | a | a |

| Accession | Landrace | *Pina-D1* | *Pinb-D1* |
|---|---|---|---|
| CWI61587 | MEX94 | a | a |
| CWI61656 | MEX94 | a | a |
| CWI61747 | MEX94 | a | a |
| CWI64775 | GTO95 | a | a |
| CWI64780 | GTO95 | a | a |
| CWI64806 | GTO95 | a | a |
| CWI64800 | GTO95 | a | e |
| CWI64806 | GTO95 | a | a |
| CWI65281 | CHIH95 | a | a |
| CWI68706 | BCS95 | a | a |
| CWI68707 | BCS95 | a | a |
| CWI68708 | BCS95 | a | a |
| CWI68709 | BCS95 | a | a |
| CWI68710 | BCS95 | a | a |
| CWI68968 | CHIH96 | a | a |
| CWI69035 | CHIH96 | a | a |
| CWI69118 | CHIH96 | a | a |
| CWI69200 | CHIH96 | a | a |
| CWI76708 | OAX93 | a | a |
| CWI76770 | OAX93 | a | a |
| CWI76845 | OAX93 | a | a |
| CWI76899 | OAX93 | a | a |
| CWI76954 | OAX93 | a | a |

## CAPÍTULO II

# CARACTERIZACIÓN DE LA DIVERSIDAD GENÉTICA Y MOLECULAR DE LAS PUROINDOLINAS (*Pina-D1* y *Pinb-D1*) EN VARIEDADES LOCALES DE TRIGOS PROVENIENTES DE ANDALUCÍA (SUR DE ESPAÑA)

**Enviado como:**

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**Resumen**

La dureza es el carácter más importante para definir la calidad del producto final en trigo harinero, la cual está directamente relacionada con la cantidad de almidón dañado producido durante el proceso de molienda. Este carácter está genéticamente controlado por los genes de las puroindolinas (*Pin*) localizados en el cromosoma 5DS. Cuarenta y cinco variedades locales de Andalucía, región al sur de España, fueron analizadas para dureza de grano y genes *Pin*. Treinta y una accesiones mostraron textura semi-dura (rango de dureza entre 44 y 55%), mientras que del resto, trece accesiones fueron duras (< 45%) y una suave (> 55%). En todas las accesiones con textura semi-dura o suave se detectaron los alelos silvestres (*Pina-D1a* y *Pinb-D1a*). Sin embargo, solamente en siete de las trece accesiones con textura dura esto pudo ser explicado mediante mutaciones bien en *Pina-D1* o *Pinb-D1*. Dos de ellas portaban el alelo nulo *Pina-D1b*. Para *Pinb-D1* se detectaron tres alelos, incluyendo *Pinb-D1b* y *Pinb-D1d*. El tercero de ellos fue provisionalmente denominado *Pinb-D1ad*, el cual está caracterizado por una transición C → T que conduce a la aparición de un codón stop prematuro, siendo este alelo extremadamente infrecuente. Estos resultados corroboran la importancia de estas variedades locales, no utilizadas frecuentemente en programas de mejoramiento, como fuentes potenciales de variabilidad genética para los loci *Pin* para el desarrollo de cultivares modernos con diferentes texturas de grano.

**Palabras clave:** trigo harinero, puroindolinas, variabilidad genética, textura de grano.

**Abstract**

In common wheat, grain hardness is the most important single trait determining wheat end-use quality, which is directly related with the amount of damaged starch produced during the milling process. This trait is genetically controlled by the puroindolines genes (*Pin*) located at the 5DS chromosome. Forty-five landraces from Andalusia, region in southern Spain, were analyzed for grain hardness and *Pin* genes. Thirty-one accessions showed semi-hard texture (hardness range between 45 and 55%), while the rest was distributed in 13 hard accessions (< 45%) and one soft-textured (> 55%). The wild alleles (*Pina-D1a* and *Pinb-D1a*) were found in all the soft- and semi-hard texture accessions. However, only seven of the thirteen hard accessions could be explained by mutations in either *Pina-D1* or *Pinb-D1*. Two of them contain the null *Pina-D1b* allele. For *Pinb-D1*, three different alleles were detected, including the *Pinb-D1b* and *Pinb-D1d*. The third of them that was tentatively named as *Pinb-D1ad* was characterized by a C → T transition that led to the appearance of a premature stop codon, being extremely rare. These results substantiate the importance of these landraces, not frequently utilized in modern breeding programmes, as potential sources of the genetic variability of *Pin* loci for the development of modern wheat cultivars with different grain textures.

**Keywords:** common wheat, puroindoline, genetic variability, grain texture

## Introduction

Wheat is one of the most important crops in the World, occupying an area of 219 million hectares with a global production of 715 million tonnes (FAOSTAT 2013). Its high adaptability to different environmental conditions, which allows it to be cultivated in almost all latitudes, together with its easy manipulation and storage, has been key in the great success of this crop. This wide distribution has generated that wheat is the base of great diversity of foods (leavened breads, flat breads, noodles, cookies, etc.), which show differential characteristics in texture, shapes, taste and aromas according to the geographical area where these wheats and products are carried out. Although the quality parameters of flour or semolina quality have been good defined for the conventional or industrial products, in many cases these values cannot extrapolated to the traditional or land products. Consequently, to define those end-use quality characteristics the grain quality properties and the processed followed to elaborate the products are very important.

Grain quality is mainly determined by dough (gluten) viscoelastic properties and grain hardness. Grain hardness is the most important single trait determining wheat end-use quality (Pomeraz and Willians 1990; Morris and Rose 1996) and globally markets classify wheat in three main hardness class: very hard durum wheat (*Triticum turgidum* ssp. *durum* Desf. em. Husn.; 2n = 4× = 28, **BBA$^{u}$A$^{u}$**), and hard or soft bread wheat (*T. aestivum* L. ssp. *aestivum*; 2n = 6× = 42, **BBDDA$^{u}$A$^{u}$**). The importance of grain hardness is due to its direct relationship with the amount of damaged starch produced during the milling process (the hard grain produce higher starch damage than the soft grain). Damaged starch can absorb almost five times more water than native starch (Kweon et al. 2011). Because of this, hard wheat is used

for bread-making that requires high water absorption for the correct development of the fermented dough, while soft wheat is preferred for cookies and pastries, which requires a flour less hydrophilic, then more water is available to the sugar to form syrup and resulting in farther dough spreading and larger diameter cookies (Guttieri et al. 2001).

Several studies indicate that grain hardness is controlled by the *Ha* (hardness) locus located at the 5DS chromosome. In this locus have been well characterised the genes encoding the grain softness protein (*Gsp-1*), together with the puroindolines (*Pina-D1* and *Pinb-D1*) that are considered the main responsible of the hardness (Morris 2002). Puroindolines (PINA and PINB) are two basic grain proteins having a tryptophan-rich-hydrophobic domain (Gautier et al. 1994), which presence in their wild type form *(Pina-D1a* and *Pinb-D1a*) leads to soft wheat texture. The lack of any of them, together with most of the mutations described either in *Pina-D1* or *Pinb-D1* leads to hard grain texture (reviewed in Morris, 2002). Some of those alleles are being associated to differences in the level of hardness and in processing and end-use quality traits (Eagles et al. 2006; Chen et al. 2013).

The landraces and obsolete cultivars possess variability for different genes, not occurring in modern cultivars (Hammer et al. 1996), which could be valuable for modern breeding programs to generate materials with novel improved properties. In a previous study, a wide collection of bread wheat landraces from Andalusia (South of Spain), were characterized for morphological characteristics and for glutenins composition (HMWGs and LMWGs) (Ayala et al. 2015). Novel alleles for *Glu-A1, Glu-D1* and *Glu-B3* loci were detected what indicated that this collection was diverse for quality traits and, therefore, it could be a good source of novel alleles of other quality related genes.

The aim of the current study was to evaluate the variability for puroindolines genes in a collection of common wheat landraces from Andalusia and characterize molecularly the alleles found.

## Material and methods

*Plant materials*

Forty-five accessions obtained from the Centro de Recursos Fitogenéticos INIA (Alcalá de Henares, Spain) and National Small Grain Collections (Aberdeen, USA), were analysed in this study (Table 1). These accessions are landraces were distributed throughout six of the eight provinces of Andalusia. These materials were grown during 2012-13 in a 1 m, one row-plot of an un-replicated trial at the CIFA-IFAPA experimental station at Córdoba, Spain, with standard agronomic practices for the region (175 Kg/ha N, 90 Kg/ha P, and 90 Kg/ha K).

*Quality analysis*

Quality analyses were performed using the methods established by the American Association of Cereal Chemists (AACC, 2000). Grain hardness (%) and grain protein content (%) were determined by a near-infrared spectroscope NIR Systems 6500 (FOSS-Tecator, HillerØd, Denmark) previously calibrated for hardness by the Particle Size Index method (PSI; AACC method 55-30, 2000) and protein content by Kjeldahl method (AACC method 46-11A, 2000). In PSI soft wheat endosperm produces higher proportion of fine particles which corresponds to higher percentages. According with the data obtained by NIRs, samples were classified in: hard (30-44%), semi-hard (45-55%) and soft (> 55%).

**Table 1.** Andalusian wheat accessions used in this study.

| Locality | Botanical variety | Genebank ID*. |
|---|---|---|
| Almería | var. *aestivum* | BGE011987; BGE013156; BGE023727 |
| | var. *aureum* (Link) Mansf. | BGE011877; BGE012113; BGE026946; BGE026947; BGE026948; BGE026949 |
| | var. *graecum* (Körn) Mansf. | BGE008216; BGE008218 |
| | var. *lutescens* (Alef.) Mansf. | BGE030918 |
| Cádiz | var. *aestivum* | BGE020363; PI 136567; PI 191017 |
| | var. *hostianum* | BGE013163 |
| Córdoba | var. *aestivum* | BGE008207; BGE008214 |
| | var. *aureum* (Link) Mansf. | BGE011825; BGE011829 |
| | var. *graecum* (Körn) Mansf. | BGE008213 |
| Granada | var. *aestivum* | BGE013207; BGE018246; BGE018254; BGE018668; PI 191215 |
| | var. *aureum* (Link) Mansf. | BGE008245; BGE012127; BGE018670; BGE020362; BGE029101; PI 191046; PI 191431 |
| | var. *graecum* (Körn) Mansf. | BGE017171 |
| | var. *hostianum* | BGE015384 |
| | var. *lutescens* (Alef.) Mansf. | BGE030917 |
| Huelva | var. *aestivum* | BGE013154; BGE018234; BGE036374; PI 191018 |
| | var. *lutescens* (Alef.) Mansf. | BGE011889; BGE012196 |
| Málaga | var. *aestivum* | BGE019332 |
| | var. *lutescens* (Alef.) Mansf. | BGE008212; BGE019331 |

* BGE: Centro de Recursos Fitogenéticos (Alcalá de Henares, Spain); and PI: National Small Grain Collection (Aberdeen, USA).

*PCR amplification and sequencing of the Pina-D1 and Pinb-D1 genes*

Genomic DNA was extracted from young leaves of single plants by the CTAB method (Stacey and Isaac 1994).

The primers designed by Lillemo et al. (2006) were used to amplify *Pina-D1* (Fw: 5′-CATCTATTCATCTCCACCTGC-3′; Rv: 5′-GTGACAGTTTATTAGCTAGTC-3′) and *Pinb-D1* genes (Fw: 5′-

GAGCCTCAACCCATCTATTCATC-3′; Rv: 5′-CAAGGGTGATTTTATTCATAG-3′). Each 15 μl reaction included 50 ng DNA, 1.5 mM $MgCl_2$, 0.2 μM of each primer, 0.2 mM dNTPs, 1.5 μl 10× PCR buffer and 0.75 U DNA polymerase (Promega). The PCR conditions included an initial denaturation step of 3 min at 94°C followed by 35 cycles as follows: 45 s at 94°C, 1 min 30 s at 60°C then 45 s at 72°C. After the 35 cycles all reactions included a final extension of 5 min at 72°C.

Amplification products were fractionated in vertical PAGE gels in a discontinuous Tris-HCl buffer system (pH 6.8/8.8) with 8% (w/v, C: 1.28%) polyacrylamide concentration. Due to the similar size of the PCR products for each gene, these were restricted by endonucleases whose effectiveness for detecting mimetic alleles had been validated (Pickering and Brave 2007; Ayala et al. 2013). The digested fragments were analysed in PAGE gels with 10% polyacrylamide concentration (C: 2.67%). In both cases, Tris/glycine buffer was used and the bands were visualized by ethidium bromide staining.

The PCR products were cloned in the pGEMT-easy vector (Promega) and the nucleotide sequences was obtained from three positive clones using the M13 universal primers in both directions on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). The sequences were analysed and compared to the sequences of common wheat available in the database (*Pina-D1a*: X69914, *Pinb-D1a*: X69912, *Pinb-D1b*: DQ363914, *Pinb-Dad*: JX187515), using the Geneious Pro software version 5.0.4 (Biomatters Ltd.).

*Statistical analysis*

The hardness values were analysed by ANOVA, and means by *Pina*/*Pinb* composition were compared using the least significant difference (LSD) test.

**Results**

A wide range of grain hardness values was found in the accessions analysed (Fig. 1), with most of the samples (31) showing semi-hard texture (hardness range between 45 and 55%). Thirteen accessions resulted to have hard grain (< 45%) while only one was soft (> 55%).

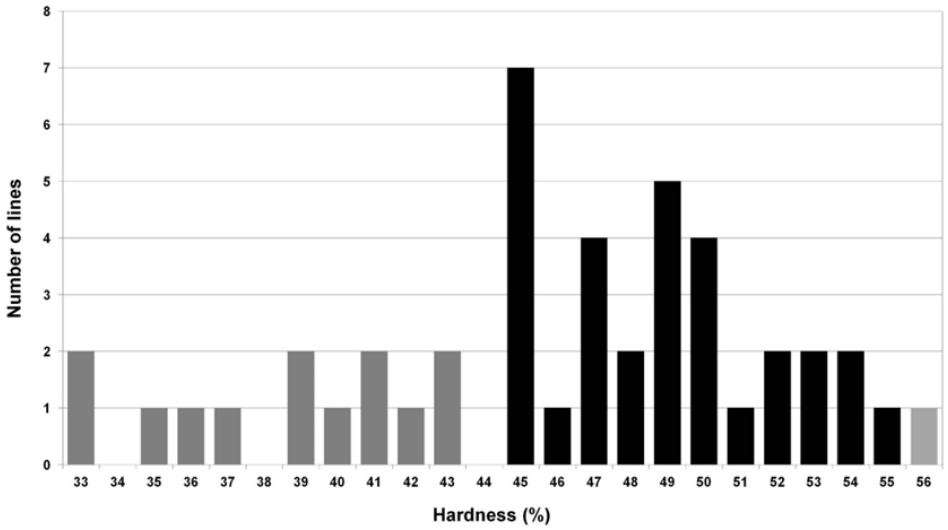


**Figure 1.** Hardness frequency distribution of the lines evaluated.

Based on this data, genotyping for *Pin-D1* genes was carried out. For *Pina-D1* gene, most of the accessions produced a PCR product with size around 524 bp (Fig. 2a), except for two accessions (BGE008216 and BGE030918) from Almería that did not show amplification product. Both accessions were classified according to the hardness value as hard grain. The possibility that the absence of amplification was due to changes in the target sequence of the primers was excluded by the PCR amplification of *Pina-D1* using another two primer pairs designed by Gautier et al. (1994) and Ikeda et al. (2010), respectively. In both cases, no amplicons were detected in these accessions (data no show), which confirmed the lack of the *Pina-D1* gene, being consequently catalogued as *Pina-D1 null* (*Pina-D1b*). On the contrary, all

accessions showed the expected amplification product with size around 597 bp for *Pinb-D1* gene, including the two accessions carrying *Pina-D1b* allele (Fig. 2b). Only one accession showed a band with lower mobility compared to the rest (Fig. 2b, lane 3).

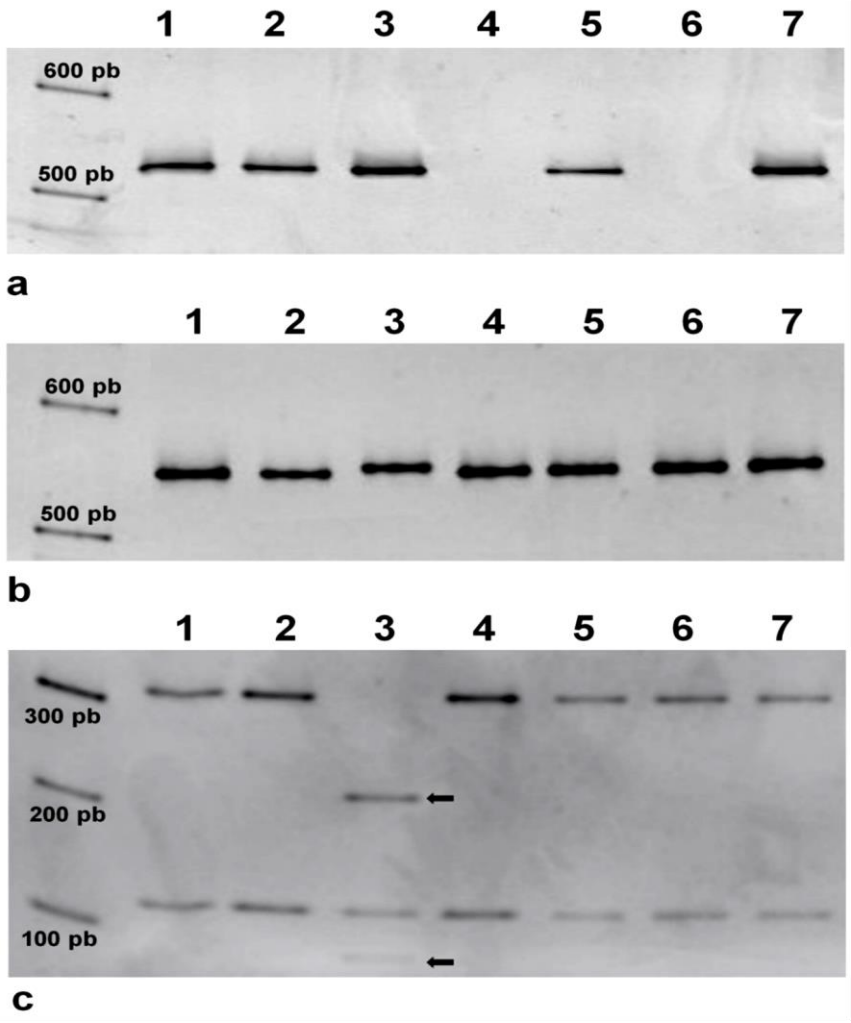


**Figure 2.** **a)** *Pina-D1* amplification; **b)** *Pinb-D1* amplification; and **c**) *Pinb-D1* digestion with *Bsr*B1 endonuclease. Lanes are as follows: *1*, BGE008245 (*Pina-D1a/Pinb-D1d*); *2*, BGE017171 (*Pina-D1a/Pinb-D1d*); *3*, BGE011877 (*Pina-D1a/Pinb-D1b*); *4*, BGE008216 (*Pina-D1b/Pinb-D1a*); *5*, BGE018668 (*Pina-D1a/Pinb-D1null*); *6*, BGE030918 (*Pina-D1b/Pinb-D1a*); and *7*, BGE013207 (*Pina-D1a/Pinb-D1a*).

Because of the use of some endonucleases (*Bsr*B1 and *Bst*XI) have been identified as an useful tool to detect further variability of these genes (Pickering and Bhave 2007), the amplifications products were digested with them. The *Bsr*B1 endonuclease allowed the identification of the *Pinb-D1b* allele in the BGE011877 accession from Almeria. This identification is due to the codon change GGC → AGC (Gly46 → Ser) that generates a restriction site for this enzyme in this *Pinb-D1* allele (Fig. 2c, lane 3). However, the cleavage with the *Bst*XI endonuclease that permit to screen for *Pinb-D1e* allele, did not detect more polymorphism.

Nevertheless, great part of the accessions with hard texture cannot be included in some of these previous groups, and showed amplicons that could be classified as wild alleles for *Pina-D1* and *Pinb-D1* genes. For this reason, both genes from the thirteen accessions showing hard texture (PSI < 45%) together with twelve random selected accessions spanning the rest of texture classes (PSI 45-56) were sequenced. These sequences were compared to the *Pina-D1a* and *Pinb-D1a* alleles from common wheat cv. Chinese Spring.

For *Pina-D1* gene, all the accessions resulted to have the wild allele (*Pina-D1a*; NCBI ID: KT885195), with the exception of the two abovementioned accessions that did not show amplification product for this gene. For *Pinb-D1* gene (Fig. 3), the sequences analysis confirmed that the BGE011877 accession contained a sequence corresponding to *Pina-D1b* allele (NCBI ID: KT885197). Besides, another three accessions from Granada (BGE008245, BGE017171 and BGE 018670), which showed hard grain texture, had a mutation in position 217 that consisted in the codon change TGG → AGG (Trp44 → Arg). This allele (NCBI ID: KT885198) was catalogued as *Pinb-D1d* by Lillemo and Morris (2000). At last, other hard grain accession (BGE018688 collected in Granada; NCBI ID: KT885199) had the change C → T in position 271, which led an early stop codon in the

deduced mature protein (Gln62 → stop). This mutation was previously reported in a sequence detected in a spelt wheat accession (NCBI ID: JX187515; Giraldo et al. unpublished results). As no name was assigned to this allele, we propose to classify it tentatively as *Pinb-D1ad* following the nomenclature of the Catalogue of Gene Symbols (McIntosh et al. 2014). All the rest of the accessions used in the sequencing analysis showed the wild allele *Pinb-D1a* (NCBI ID: KT885196), including the three accessions BGE012127, BGE036374 and BGE018254 from Granada, which have showed hard endosperm in grain hardness analysis.

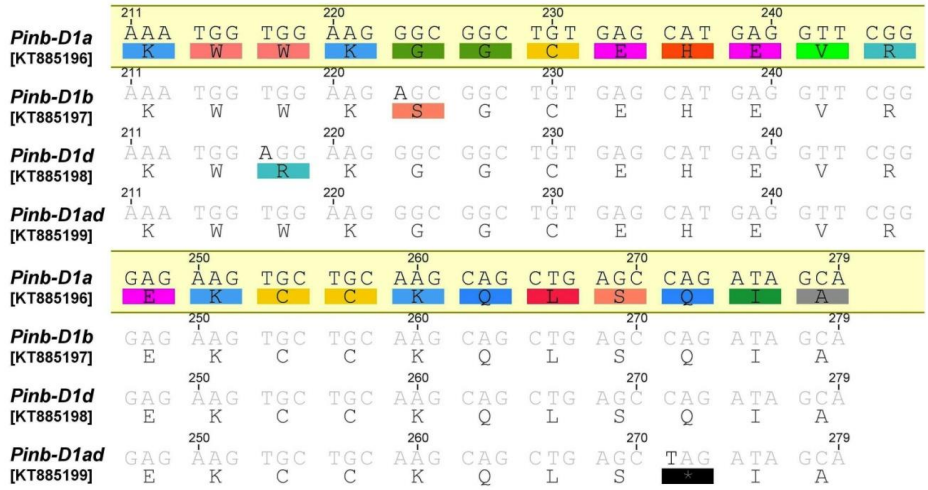


**Figure 3.** Alignment of DNA sequence of the *Pinb-D1* genes detected in the current study.

According with the variation found, five haplotypes were detected within of these accessions, being the *Pina-D1a*/*Pinb-D1a* haplotype the most frequent with 84.4% (Table 2). The ANOVA analysis confirmed that the presence of any allele different to the wild ones generated materials with hard texture (hardness < 39%). However, some of the accessions with wild haplotype showed hardness values that should be classified as hard type (hardness between 39 and 43%).

**Table 2.** Allelic composition for puroindolines genes and hardness values of the lines evaluated

| *Pina-D1* | *Pinb-D1* | N | Hardness (%) | Range |
|---|---|---|---|---|
| *a* | *a* | 38 | 47.8a | 39-56 |
| *a* | *null* | 1 | 39.0b | - |
| *a* | *b* | 1 | 37.0b | - |
| *a* | *d* | 3 | 34.7b | 33-36 |
| *b* | *a* | 2 | 37.0b | 33-41 |

Means with the same letter are not significant different at 95%.

## Discussion

In Andalusia, wheat has been cultivated since ancient times and used for the manufacturing of diverse products, which require specific quality traits. In the current study, a collection of Andalusian traditional varieties/landraces was analysed for grain hardness and characterized at the molecular level by puroindoline composition (*Pina-D1* and *Pinb-D1*). The same collection showed high diversity for glutenins in a previous study (Ayala et al. 2015). The distribution of grain hardness was continuous with a range of values spanning from 33 to 56 in the PSI test. Most of the lines (31) resulted to have semi-hard texture, with also thirteen lines showing hard grain and only one soft. These results are in agreement with the traditional main use of wheat in the region, which is the elaboration of different kind of specialities breads as 'Pan de Alfacar'. These breads require doughs with high-medium water absorption and medium/weak gluten strength (Ayala et al. 2015).

Of the thirteen accessions showing hard grain texture, in seven of them hard texture could be explained by mutations in either *Pina-D1* or *Pinb-D1*, in agreement with results published earlier (Giroux and Morris 1997; Lillemo et al. 2006; Ayala et al. 2013). The null *Pina-D1b* allele was identified in two of the thirteen hard lines. The presence of this allele is common in

germplasm with diverse geographical origin (Eagles et al. 2006; Lillemo et al. 2006; Chen et al. 2007) and was also reported in Mexican landraces derived from Spanish wheats brought to the new continent by the colonist (Ayala et al. 2013). It is associated with harder endosperm than other *Pin* alleles as *Pinb-D1b* (Giroux et al. 2000; Chen et al. 2013), although in the current study not significant differences in grain hardness were detected among the lines carrying different *Pin-D1* alleles. This was probably due to the limited number of lines with differential *Pin-D1* alleles and to the different genetic background of the accessions that avoids detecting small differences due to different *Pin-D1* alleles.

In the remaining eleven hard grain lines, *Pina-D1* and *Pinb-D1* sequencing analysis were carried out to try to find the genetic reason of the hard endosperm texture. No polymorphism was detected for *Pina-D1*, and all the accessions presented the wild allele *Pina-D1a*. For *Pinb-D1*, three different mutations compared to the wild-type allele *Pinb-D1a* were detected. The first of them, a G → A transition in position 223 (Gly-46 to Ser-46) that was first reported by Giroux and Morris (1997) and named *Pinb-D1b*, was detected in one accession of the current study by the use of endonuclease *Bsr*B1 according to Pickering and Bhave (2007). Other three accessions had a mutation described first by Lillemo and Morris (2000) in hard wheats from Northern Europe and named *Pinb-D1d*, which consisted of a tryptophan to arginine change at position 44. This change is inside the tryptophan rich domain of the protein and will probably affect the interaction of PINB with the lipid membrane and, therefore, its functionality, leading to hard grain texture. The last mutation detected (a C → T transition that led to the appearance of a premature stop codon) was the same to the previously described in a spelt wheat accession included in the NCBI database (JX187515). We tentatively named this allele as *Pinb-D1ad*, which is extremely are. Because of this, these traditional varieties, not frequently utilized in

modern breeding programmes, could be used to widen the genetic variability of Pin loci in modern wheats.

However, in six of the accessions showing hard texture and in seven more semi-hard ones in which *Pina-D1* or *Pinb-D1* were also sequenced, no mutation was found in *Pina-D1* or in *Pinb-D1*. Grain hardness has been said to be largely controlled by genetic factors but it can also be affected by environmental conditions (Huebner and Gaines 1992, Mikulíková 2007, Turnbull and Rahman 2002) and other grain characteristics as protein content. In the current study the grain protein content (ESM 1) was in overall medium, so that factor cannot be used to explain the hard grain phenotypes in genetically soft accessions. Therefore, it seems it is possible to have genetically soft wheat which is physically semi-hard by changing environmental and drying conditions (Hoseney 1987). Consequently *Pin-D1* genetic classification alone may not necessarily describe the physical characteristics of the grain, although is true that all the accessions not having the wild genotype for *Pin-D1* genes had all hard texture.

In conclusion, although this collection of old Andalusian landraces has no novel alleles of the puroindoline genes, but include one very rare allelic variant (*Pinb-D1ad*). The results therefore provide evidence of the importance of these genetic materials, not frequently utilized in modern breeding programmes, as sources of genetic diversity for use in breeding programmes.

**Acknowledgements**

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**Table supplementary**

| NUMBAN | NOMLOC | Locality | Hardness (%) | Protein | *Pina-D1* | *Pinb-D1* |
|---|---|---|---|---|---|---|
| BGE013207 | Sierra Nevada-A | Granada | 45 | 16.3 | *a* | *a* |
| BGE008216 | Trigo de Almería-A | Almería | 33 | 12.9 | *b* | *a* |
| BGE008218 | Trigo de Almería-B | Almería | 49 | 12.4 | *a* | *a* |
| BGE011877 | Desconocido de Almería | Almería | 37 | 12.1 | *a* | *b* |
| BGE011987 | Candeal compacto | Almería | 51 | 13.8 | *a* | *a* |
| BGE012113 | Candeal mocho | Almería | 47 | 11.7 | *a* | *a* |
| BGE013156 | Rojo | Almería | 47 | 14.5 | *a* | *a* |
| BGE023727 | Desconocido de Vélez Rubio | Almería | 45 | 13.7 | *a* | *a* |
| BGE026946 | Pichi-Las tres Villas | Almería | 45 | 14.1 | *a* | *a* |
| BGE026947 | Pichi-Chirivel | Almería | 54 | 12.2 | *a* | *a* |
| BGE026948 | Pichi-Gergal | Almería | 46 | 13.6 | *a* | *a* |
| BGE026949 | Pichi-Alcontar | Almería | 45 | 12.5 | *a* | *a* |
| BGE030918 | Pichi-Senes | Almería | 41 | 12.5 | *b* | *a* |
| BGE013163 | Barbilla-A | Cádiz | 39 | 14.5 | *a* | *a* |
| BGE020363 | Trigo de Grazalema | Cádiz | 40 | 13.9 | *a* | *a* |
| BGE008207 | Trigo de Córdoba-A | Córdoba | 47 | 13.7 | *a* | *a* |
| BGE008213 | Blanquillo | Córdoba | 55 | 14.3 | *a* | *a* |
| BGE008214 | Trigo de Córdoba-B | Córdoba | 48 | 15.6 | *a* | *a* |
| BGE011825 | Ratón de Belalcazar | Córdoba | 50 | 13.6 | *a* | *a* |
| BGE011829 | Rabón de Hinojosa | Córdoba | 52 | 12.4 | *a* | *a* |
| BGE008245 | Trigo de Granada-A | Granada | 33 | 11.4 | *a* | *d* |
| BGE012127 | Candeal de Sierra Nevada | Granada | 41 | 13.7 | *a* | *a* |
| BGE015384 | Trigo del Gobierno-A | Granada | 45 | 13.1 | *a* | *a* |
| BGE017171 | Mocho | Granada | 35 | 11.5 | *a* | *d* |
| BGE018246 | Sierra Nevada cañihueca | Granada | 45 | 15.3 | *a* | *a* |
| BGE018254 | Sierra Nevada-B | Granada | 43 | 14.5 | *a* | *a* |
| BGE018668 | Desconocido de Puebla de Don Fadrique | Granada | 39 | 13.1 | *a* | *null* |
| BGE018670 | Trigo de Soportar | Granada | 36 | 10.9 | *a* | *d* |

| NUMBAN | NOMLOC | Locality | Hardness (%) | Protein | *Pina-D1* | *Pinb-D1* |
|---|---|---|---|---|---|---|
| BGE020362 | Candeal | Granada | 49 | 11.8 | *a* | *a* |
| BGE029101 | Trigo Granada 24 | Granada | 56 | 12.7 | *a* | *a* |
| BGE030917 | Trigo del Gobierno-B | Granada | 52 | 13.9 | *a* | *a* |
| BGE011889 | Rojo pelón | Huelva | 43 | 11.7 | *a* | *a* |
| BGE012196 | Pelón blanco | Huelva | 49 | 12.5 | *a* | *a* |
| BGE013154 | Barbilla blanca | Huelva | 49 | 13.6 | *a* | *a* |
| BGE018234 | Barbilla roja | Huelva | 45 | 13.8 | *a* | *a* |
| BGE036374 | Trigo barbilla rerbero | Huelva | 42 | 11.7 | *a* | *a* |
| BGE008212 | Trigo de Málaga | Málaga | 54 | 15.1 | *a* | *a* |
| BGE019331 | Desconocido de Málaga-A | Málaga | 53 | 13 | *a* | *a* |
| BGE019332 | Desconocido de Málaga-B | Málaga | 49 | 14.4 | *a* | *a* |
| PI-191017 | Barbilla de Cádiz | Cádiz | 50 | 12.4 | *a* | *a* |
| PI-191018 | Barbilla de Huelva | Huelva | 48 | 12.6 | *a* | *a* |
| PI-191046 | Candeal de Granada | Granada | 50 | 13.8 | *a* | *a* |
| PI-191215 | Sierra Nevada-B | Granada | 47 | 16 | *a* | *a* |
| PI-191431 | Mocho Blanco del Valle | Granada | 53 | 14.1 | *a* | *a* |
| PI-136567 | Barbilla-B | Cádiz | 50 | 13.1 | *a* | *a* |

# CAPÍTULO III

# CARACTERIZACIÓN DE LA DIVERSIDAD FENOTÍPICA (MORFOLOGÍA DE PLANTA Y GRANO) Y GENOTÍPICA (ALELOS DE GLUTENINA EN LOS LOCUS *Glu-1* Y *Glu-3*) EN VARIEDADES LOCALES DE TRIGOS (*Triticum aestivum*) DE ANDALUCÍA (SUR DE ESPAÑA)

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**Resumen**

Cuarenta y seis variedades locales de Andalucía, región sur de España, se caracterizaron para la morfología de la planta, espiga y grano y para la composición de HMWGs y LMWGs. Se identificaron cinco variedades botánicas entre estos materiales basados en cuatro rasgos morfológicos. De acuerdo con el color del grano, nueve accesiones mostraron heterogeneidad. Los rasgos morfológicos restantes mostraron una amplia variación. El análisis para HMWGs y LMWGs mostró una gran variación en estas líneas, pudiéndose encontrar hasta 77 genotipos en estas 46 accesiones. El análisis de HMWGS, detectó un alelo nuevo de *Glu-A1* (subunidad 1'") presente en cuatro líneas (BGE012113 var *aureum*; BGE013163 var *hostianum*; BGE012196 y BGE026948 var *lutescens*.), otro para *Glu-D1* (subunidades 3*+T2) sólo presente en una línea derivada del BGE036374, var. *aestivum*. Para las LMWGs se detectó un nuevo alelo para el locus *Glu-B3*. La variación alélica en el *Glu-1* y *Glu-3* mostró un claro riesgo de erosión genética debido a la baja frecuencia de algunos alelos. La caracterización de estas variedades locales podría permitir la evaluación de la relación entre los diversos usos de estas harinas y la composición de sus proteínas de almacenamiento de las semillas. La recuperación de variedades antiguas o razas locales es significativa para los agricultores que pueden sembrar los materiales y beneficiarse de su valor potencial en el mercado.

**Palabras claves:** Electroforesis; Diversidad genética; Glutenina; Características morfológicas; *Triticum aestivum*

**Abstract**

Forty-six landraces from Andalusia, region in southern Spain, were characterized for plant, spike and grain morphology and for HMWGs and LMWGs composition. Up to five botanical varieties were identified among these materials based in four morphological traits. According to grain colour, nine accessions showed heterogeneity. The remaining morphological traits showed wide variation. The analysis for HMWGs and LMWGs showed that the variation of these lines indicates that up to 77 genotypes could be found in these 46 accessions. The analysis for the HMWGS, detected a novel allele for *Glu-A1* (subunit 1'") found in four lines (BGE012113 var. *aureum*; BGE013163 var. *hostianum*; BGE012196 and BGE026948 var. *lutescens*), and for *Glu-D1* (subunits 3*+T2) present in only one line derived from the BGE036374, var. *aestivum*. For the LMWGs a new allele for the *Glu-B3* locus was detected. The allelic variation at the *Glu-1* and *Glu-3* showed a clear risk of genetic erosion due to the low frequency of some alleles. The characterization of these landraces could permit the evaluation of the relationship between the uses of these flours and the composition of their seed storage proteins. The recovery of old varieties or landraces is significant for farmers who can plant the materials and benefit from their potential value in the marketplace.

**Keywords:** Electrophoresis; Genetic diversity; Glutenin; Morphological trait; *Triticum aestivum*

## Introduction

Andalusia is a region in southern Spain where traditional wheat products made with flour from traditional, locally grown, old varieties can still be found. Until recently, such products were made with flour derived from traditional, locally grown, old varieties. However, since the 1960s, these old cultivars have been gradually substituted with modern, high yielding varieties, mostly of CIMMYT origin, particularly in the west of the region in the Guadalquivir River Valley. The use of flour derived from modern cultivars has resulted in changes to the properties of traditional wheat products. The products from the Protected Geographical Indication (PGI) 'Pan de Alfacar' (O.J.E.U 2013) are a good example of this, which had been traditionally performed with wheat varieties of weak gluten. In recent times, a significant revival of classical cuisine and culinary techniques throughout Europe has led to renewed interest in recovering old wheat varieties for use in manufacturing traditional foods as local breads or biscuits. In turn, this has led to renewed interest in developing and breeding new wheat varieties with better agronomical traits but with similar quality properties to the traditional ones. Unfortunately, much old material, which could provide a valuable resource, no longer exists on farms and is only found in Genebanks (Esquinas-Alcázar 2005). Even in Genebanks the information available on traditional varieties is often scarce or fragmentary. Before re-introducing them for use in national wheat breeding programmes, characterization of these old varieties is fundamental (Brown et al. 1989).

During the nineteenth century, wheat variability in Spain was studied by Spanish botanists Mariano Lagasca and Simón de Rojas Clemente and featured in their unpublished work, «*Ceres Hispanica*», which detailed the plants in a herbarium collection (Téllez Molina and Peña 1952). In this

herbarium, the common wheat (*Triticum aestivum* L. ssp. *aestivum*; syn. *T. vulgare* Vill.) was apparently represented by 466 items grouped into 22 botanical varieties (eight from the *mutica* Group, and 14 from the *aristata* Group), which were classified according to four morphological traits of the spike and grain: glumes colour; hairiness of glumes; grain colour and whether or not awns were present. This classic study permits comparison of the variability present in the nineteenth century at a time when those varieties were still widely used in Spain, to the present when such botanical types are found only conserved in Genebanks. Their conservation permits study to determine the extent of homogeneity and variability – as well as synonymies and homonymies – within these old varieties or landraces.

Intra-varietal variability can also be measured by analysing the endosperm storage proteins profile. These proteins are divided into two main groups (gliadins and glutenins) according to their molecular characteristics (Payne 1987). Glutenins are also divided into high-molecular-weight (HMW-Gs) and low-molecular-weight (LMW-Gs) subunits (Singh and Shepherd 1988; Pogna et al. 1990). The HMW-Gs are encoded at the *Glu-1* loci located on the long arm of homoeologous group-1 chromosomes (Payne 1987); whereas the LMW-Gs are encoded at the *Glu-3* loci on the short arm of the same homoeologous group. In the seed, these proteins serve as source of amino acids during germination, which has allowed these genes to mutate without affecting the viability of the plant, generating great variability inside the proteins, permitting their use as efficient molecular markers for the evaluation of the genetic diversity (Gepts 1990). Furthermore, these proteins have a direct bearing on the technological properties of wheat. It is well documented that glutenins have a major impact on processing and end-use quality due to their contributions to dough strength and extensibility (see Wrigley et al. 2006 for a review).

Over the last decade, we have conducted several studies on the genetic diversity of both morphological traits and endosperm storage proteins in ancient Spanish wheat, at three ploidy levels: diploid (Alvarez et al. 2006); tetraploid (Pflüger et al. 2001; Alvarez et al. 2007; Caballero et al. 2008; Carmona et al. 2010;) and hexaploid (Caballero et al. 2001, 2004). Recently, this research has been extended to include common Mexican wheat landraces, which contain the last trace of common wheat carried by the Spanish to Mexico during the sixteenth-eighteenth centuries (Caballero et al. 2010). The evaluation of the traditional wheat varieties and ancient wheat germplasm from Andalusia stored in Genebanks could be of great importance in the development of new materials, which could be used to aid the revival of the production of traditional foods.

The aim of the current study was to evaluate the phenotypic (morphological characteristics) as well as the gluten protein-related genotypic (HMWGs and LMWGs) composition in a collection of common wheat landraces from Andalusia.

## Materials and methods

*Plant materials*

Forty-six accessions obtained from the Centro de Recursos Fitogenéticos INIA (Alcalá de Henares, Spain) and National Small Grain Collections (Aberdeen, Idaho, USA), were analysed in this study (Supplementary Table 1). These materials were distributed throughout seven of the eight provinces of Andalusia (Fig. 1). These plants were grown during 2012-13 in a 1 m, one row-plot of an unreplicated trial at the CIFA-IFAPA experimental station at Córdoba, Spain, with standard agronomic practice for the region (175 Kg/ha N, 90 Kg/ha P, and 90 Kg/ha K).

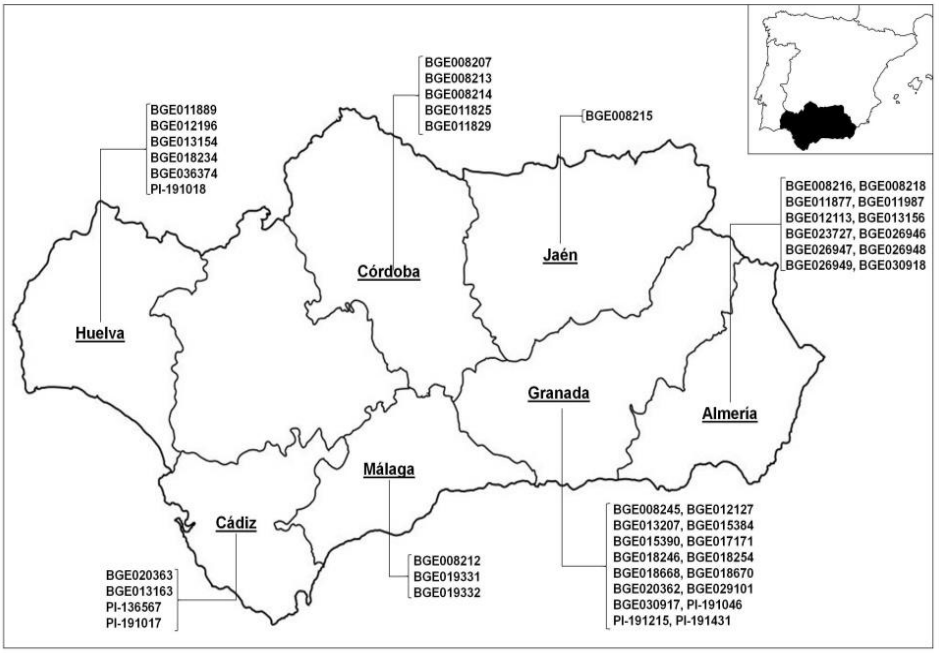


**Figure 1.** Distribution of the bread wheat accessions evaluated in this study.

*Morphological characterization*

Five plants of each accession were individually analysed to detect their possible heterogeneity. Eleven morphological traits from the descriptors proposed by the International Union for the Protection of New Varieties of Plants (UPOV 1994) were used to evaluate each accession. These traits were: plant height; section of stem; hairiness in spike neck; spike shape; spike density; glumes colour; glumes hairiness; presence or absence of awns; glume-shoulder width; glume-shoulder shape and grain colour.

Four out of these traits (glumes colour, hairiness in glumes, presence or absence of awns and grain colour) were used for grouping the lines by botanical varieties as indicated by Lagasca and Rojas Clemente (Téllez Molina and Peña 1952).

*Protein extraction and electrophoretic analysis*

A sample of 40 mg of wheat flour was used to obtain glutenin and gliadin fractions, according to (Peña et al. 2004). These proteins were fractionated by electrophoresis in vertical SDS-PAGE slabs in a discontinuous Tris-HCl-SDS buffer system (pH: 6.8/8.8) with gels at a polyacrylamide concentration of 8% (w/v, C = 1.28%). LMW glutenin subunits were analysed by 10% gels (w/v, C: 1.28%). The Tris-HCl/glycine buffer system of Laemmli (1970) was used. Electrophoresis was performed at a constant current of 30 mA/gel at 18°C for 30 minutes after the tracking dye migrated off the gel. To elucidate some allelic HMWGs variants, a further analysis by SDS-PAGE was performed, using 8% gels containing 4M urea as reported by Lafiandra et al. (1993). Gels were stained overnight with 12% (w/v) trichloroacetic acid solution containing 5% (v/v) ethanol and 0.05% (w/v) Coomassie Brillant Blue R-250. De-staining was carried out with tap water.

The HMWGs alleles were designated according to Payne and Lawrence (1983), while for the LMWGs classification the systems of Singh et al. (1991) and Jackson et al. (1996) were used.

*Statistical analysis*

Allelic frequency for all the measured traits was calculated. The classification of Marshall and Brown (1975) was used for evaluating the distribution of alleles or categories. To assess potential genetic erosion, the effective number of alleles per locus (*Ne*) and Nei's diversity index (*He*) were measured (Nei 1972, 1973).

For the *Glu-A1*, *Glu-B1*, *Glu-D1*, *Glu-A3*, *Gli-B1/Glu-B3* and *Glu-D3* loci, the genetic diversity over all region (*Ht*) together with the average genetic diversities within (*Hs*) and among (*Dst*) provinces were calculated according to Nei (1973). The relative magnitude of genetic differentiation

among provinces, *Gst*, is estimated as *Dst*/*Ht*. Popgene software version 1.32 (Yeh et al. 1997) was used for the estimation of the above parameters.

## Results

*Morphological characterization*

According to criteria used by Lagasca and Rojas Clemente in their herbarium «*Ceres Hispanica*» (Téllez Molina and Peña 1952), all lines showed white glumes – 44 of them were glabrous and only two had pubescent glumes (Table 1). These last two accessions also showed awns, whereas 21 out of 44 accessions with glabrous glumes were awnless. When grain colour was analysed, nine accessions showed heterogeneity for this characteristic, seven in the *mutica* Group of accessions with glabrous glumes and two in the *aristata* Group. In total, 22 and 33 accessions showed at least one plant with white or red grain, respectively. Based on the morphological results, the lines were grouped into five botanical varieties: two of the *mutica* Group (var. *aureum* and var. *lutescens*) and three of the *aristata* one (var. *graecum*, var. *aestivum* and var. *hostianum*) (Table 1). When the rest of the morphological traits were considered, these 55 lines were grouped into two sets: the first with 37 lines obtained from the same number of accessions from the Genebank; and the second with 18 obtained from nine accessions with differences in grain colour.

Four of these heterogeneous accessions were collected in Almeria and were catalogued as 'Pichi', a landrace widely used in this zone. In all cases, the accessions present two types, according to grain colour. This was also detected in another three accessions collected in Granada, together with one in Cordoba and other in Cadiz.

The additional characterization of these 55 lines by three plant traits and four spike traits is shown in Table 2. Independently of the variation detected among the different accessions, all the lines showed intra-variation

for these seven traits. The three plant traits permitted the grouping of these materials into ten different sets. They were the lines with very high plant height, medium stems and without hairiness in spike neck, the most frequent (21 lines from five botanical varieties), following the lines with similar characteristics but thin stems (14 lines from four botanical varieties). Of the other eight sets, five were represented by only one line, while the other three had five lines in each.

**Table 1.** Evaluated common wheat landraces grouped according to the classification of Lagasca and Rojas Clemente.

| Glumes type | Awn | Grain colour | Botanical variety | Genebank ID[a]. |
|---|---|---|---|---|
| Glabrous | Absent | White | var. *aureum* (Link) Mansf. | **BGE008245**; BGE011825; BGE011829 BGE011877; BGE012113; BGE012127 **BGE018670**; BGE020362; **BGE026946** **BGE026947**; **BGE026948**; **BGE026949** **BGE029101**; PI-191046; PI-191431 |
| | | Red | var. *lutescens* (Alef.) Mansf. | BGE008212; **BGE008245**; BGE011889 BGE012196; **BGE018670**; BGE019331 **BGE026946**; **BGE026947**; **BGE026948** **BGE026949**; **BGE029101**; BGE030917 BGE030918 |
| | | White | var. *graecum* (Körn) Mansf. | BGE008213; **BGE008214**; BGE008215 BGE008216; BGE008218; BGE017171 **BGE020363** |
| | Present | Red | var. *aestivum* | BGE008207; **BGE008214**; BGE011987 BGE013154; BGE013156; BGE013207 BGE018234; BGE018246; BGE018254 BGE018668; BGE019332; **BGE020363** BGE023727; BGE036374; PI-136567 PI-191017; PI-191018; PI-191215 |
| Pubescent | Present | Red | var. *hostianum* (Clem.) Mansf. | BGE013163, BGE015384 |

[a]BGE: Centro de Recursos Fitogenéticos (Alcalá de Henares, Spain); and PI: National Small Grain Collection (Aberdeen, USA).
The accessions that showed heterogeneity appeared in black.

**Table 2.** Frequencies and genetic diversity among the evaluated accessions of common wheat

| Trait | Class | Total (n=55) | var. *aureum* (n=15) | | var. *lutescens* (n=13) | | var. *graecus* (n=7) | | var. *aestivum* (n=18) | | var. *hostianum* (n=2) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Pure (n=8) | Mix (n=7) | Pure (n=6) | Mix (n=7) | Pure (n=5) | Mix (n=2) | Pure (n=16) | Mix (n=2) | |
| Plant height | medium (81-100 cm) | 2 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| | high (101-120 cm) | 8 | 1 | 2 | 1 | 2 | 1 | 0 | 1 | 0 | 0 |
| | very high (> 120 cm) | 45 | 7 | 5 | 5 | 5 | 2 | 2 | 15 | 2 | 2 |
| Section of stem | thin | 25 | 6 | 3 | 2 | 3 | 1 | 1 | 7 | 1 | 1 |
| | medium | 30 | 2 | 4 | 4 | 4 | 4 | 1 | 9 | 1 | 1 |
| Hairiness in spike neck | null | 42 | 5 | 7 | 3 | 7 | 4 | 1 | 12 | 1 | 2 |
| | medium | 13 | 3 | 0 | 3 | 0 | 1 | 1 | 4 | 1 | 0 |
| Spike shape | claviform | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| | fusiform | 54 | 8 | 7 | 6 | 7 | 5 | 2 | 15 | 2 | 2 |
| Spike density | laxa | 6 | 4 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| | medium | 22 | 3 | 4 | 0 | 4 | 0 | 2 | 7 | 2 | 0 |
| | dense | 17 | 1 | 1 | 4 | 1 | 3 | 0 | 7 | 0 | 0 |
| | very dense | 10 | 0 | 2 | 1 | 2 | 2 | 0 | 2 | 0 | 1 |
| Glume-shoulder width | absent / very narrow | 2 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| | narrow | 21 | 4 | 1 | 1 | 1 | 1 | 2 | 7 | 2 | 2 |
| | medium | 29 | 4 | 5 | 4 | 5 | 2 | 0 | 9 | 0 | 0 |
| | broad | 3 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| Glume-shoulder shape | sloping | 2 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| | slightly sloping | 24 | 4 | 2 | 1 | 2 | 1 | 2 | 8 | 2 | 2 |
| | straight | 27 | 4 | 5 | 4 | 5 | 2 | 0 | 7 | 0 | 0 |
| | elevated | 2 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |

Pure: homogeneous accessions; Mix: heterogeneous accessions.

For the spike traits, only one line of var. *aestivum* (BGE011987) showed a spike shape different to the rest. The variation was also low for the glume-shoulder width and shape, because, although four classes were detected for both traits, only two of them in each case represented the upper to 90% of the variation (Table 2). For the fourth trait (spike density), the distribution of the frequencies among the four classes detected was more balanced.

*HMWGs and LMWGs composition*

The fifty-five lines were analysed for their glutenin composition (Table S1). This analysis showed that only 24 out of 37 lines that were homogeneous for morphological traits were also homogeneous for heir HMWGs and LMWGs composition. For the other 13 lines, in eleven cases two different combinations were found, whereas one line (BGE002813) showed three combinations and other one (BGE036374) have four combinations. On the contrary, in the lines showing variable morphology, the additional variation based on glutenin composition was low, and only four lines showed more than one combination, two in red grain variant (BGE018670 and BGE026946) and two in white grain one (BGE026947 and BGE026949). Consequently, the combined use of the morphological traits and glutenin composition indicated that up to 77 genotypes could be found in the 46 accessions.

For the HMWGs, most of the alleles found in these accessions have been previously described (McIntosh et al. 2013), with exception of one *Glu-A1* allele (subunit 1"") detected in four lines (BGE012113 var. *aureum*; BGE013163 var. *hostianum*; BGE012196 and BGE026948 var.*lutescens*), and one *Glu-D1* allele (subunits 3*+T2) present in only one line derived from the BGE036374, var. *aestivum*. Because the apparent mobility of these novel subunits does not permit their unambiguous classification, an alternative separation in SDS-PAGE gel with 4 M urea was used to discriminate these

alleles (Fig. 2). The addition of 4M urea to the gel generated an important change in the mobility of novel subunits 1''' (lanes 3 and 4), so while in gel without urea this subunit presented an intermediate mobility between the subunits 1 and 2* (lanes 1 and 5-6, respectively); in gel with urea showed a size lower than the subunit 2* and similar to the Bx subunits 7 (lane 6) or 13 (lane 3). A similar scenario was apparent in subunit 3* (lane 6) which in normal gel showed a size similar to the subunits 3 or 4 (lanes 4-5 and 2, respectively), while in gel with urea presented a size lower and similar to subunit 1 (lane 1).

**Table 3.** Allelic frequencies for the *Glu-1* and *Glu-3* loci among the evaluated accessions of common wheat

| Locus | Allele[a] | Subunits | % | *Ne* | *He* | Locus | Allele[b] | % | *Ne* | *He* |
|---|---|---|---|---|---|---|---|---|---|---|
| *Glu-A1* | *a* | 1 | 22.08 | 2.22 | 0.55 | *Glu-A3* | *b* | 3.90 | 3.72 | 0.73 |
| | *b* | 2* | 62.34 | | | | *c* | 15.58 | | |
| | *c* | null | 10.39 | | | | *d* | 16.88 | | |
| | *new* | 1''' | 5.19 | | | | *e* | 11.69 | | |
| *Glu-B1* | *a* | 7+null | 11.69 | 2.70 | 0.63 | | *f* | 44.16 | | |
| | *b* | 7+8 | 11.69 | | | | *g* | 7.79 | | |
| | *c* | 7+9 | 2.60 | | | *Gli-B1* | *b/b* | 5.19 | 3.95 | 0.75 |
| | *d* | 6+8 | 1.30 | | | */Glu-B3* | *b/b'* | 1.30 | | |
| | *e* | 20x+20y | 57.14 | | | | *c/g* | 7.79 | | |
| | *f* | 13+16 | 11.69 | | | | *e/f* | 2.60 | | |
| | *i* | 17+18 | 3.90 | | | | *f/g* | 6.49 | | |
| *Glu-D1* | *a* | 2+12 | 77.92 | 1.59 | 0.37 | | *g/f* | 3.90 | | |
| | *b* | 3+12 | 12.99 | | | | *b/d* | 15.58 | | |
| | *d* | 5+10 | 7.79 | | | | *i/c'* | 2.60 | | |
| | *new* | 3*+T2 | 1.30 | | | | *m/i* | 45.45 | | |
| | | | | | | | *q/b* | 7.79 | | |
| | | | | | | | *new* (*) | 1.30 | | |
| | | | | | | *Glu-D3* | *a* | 32.47 | 2.67 | 0.63 |
| | | | | | | | *b* | 48.05 | | |
| | | | | | | | *c* | 19.48 | | |

[a] According to Payne and Lawrence (1993)
[b] According to Singh et al. (1991) and Jackson et al. (1996)

The allelic frequencies detected for the *Glu-1* and *Glu-3* loci are shown in Table 3. In all cases, it is possible to observe the presence of hegemonic

alleles as *Glu-A1b* (subunit 2*) with 48 lines, *Glu-B1e* (subunits 20+20) with 44, or *Glu-D1a* (subunits 2+12) with 60, together with other rare ones (frequency ≤ 5%) or very rare ones (frequency ≈ 1% or lower). The data obtained for the effective number of alleles (*Ne*) showed that in the three cases there is clear danger of genetic erosion, more important for the *Glu-D1* locus (Table 3). In this same sense, the genetic diversity (*He*) was lower for the *Glu-D1* locus. When the three loci were evaluated together, the most frequent combination was: 2*, 20+20, 2+12 that appeared in 24 out of the 77 lines. This combination is associated to low gluten quality (score = 6) according with the *Glu-1* quality score designed by Payne (1987).

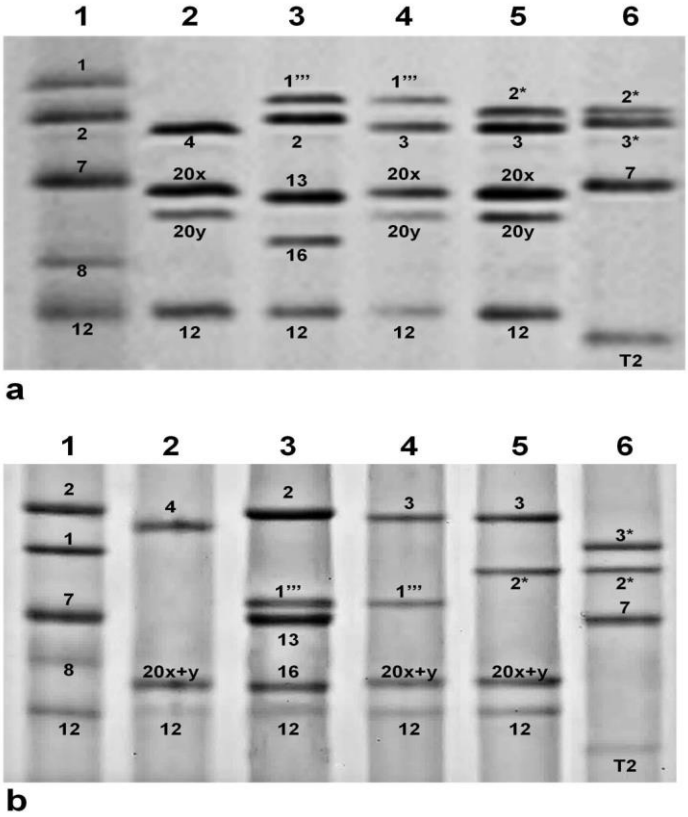


**Figure 2.** SDS-PAGE without (a) and with (b) 4M urea of some HMW subunits detected. A, *Lanes* are as follow: *1* cv. Pitic; *2* cv. Zhongyu 415; *3* BGE013163; *4* BGE012113; *5* BGE026946; and *6* BGE036374.

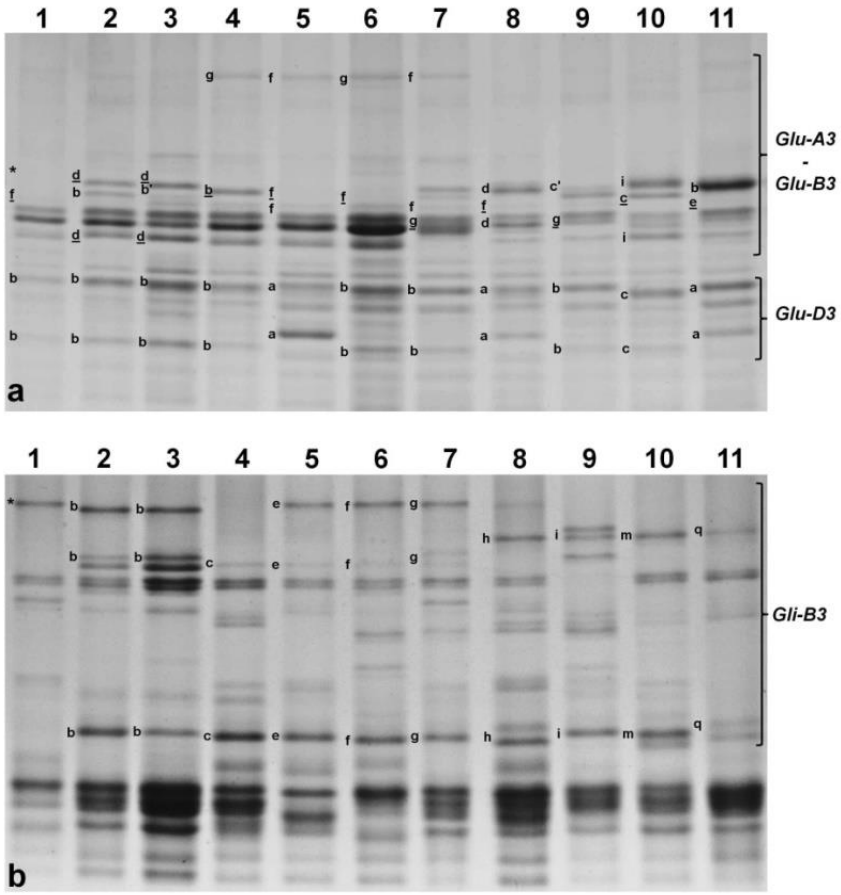


**Figure 3.** SDS-PAGE separation of the LMW subunits (a) and gliadins (b). *Lanes* are as follow: *1* BGE012113; *2* BGE030918-2; *3* BGE030918-1; *4* BGE008216-1; *5* BGE026948-1; *6* BGE026947-3; *7* BGE013163; *8* BGE013207; *9* BGE011877; *10* BGE008245-1; and *11* BGE011889. Inside the *Glu-A3*/*Glu-B3* block, the *Glu-A3* allele appears *underlined*. The new *Glu-B3* allele was marked with an *asterisk*.

For the LMWGs, the alleles detected in these materials were previously described by Singh et al. (1991) and Jackson et al. (1996), with exception of a new allele for the *Glu-B3* locus (Fig. 3, lane 1). The *Ne* values were almost 50% of the number of alleles detected, with the exception of the *Glu-D3* locus (Table 3). This is the consequence of the presence of the hegemonic alleles together with others with lower frequencies, which could be easily erased by genetic drift. For the *Glu-A3* locus, the allele *Glu-A3f* appeared in

34 lines, being less frequent than the rest of the alleles, detaching only the allele *Glu-A3d* with 13 lines and the allele *Glu-A3c* with 11 ones. Similar data were found in the *Glu-B3* locus where the 11 alleles detected showed low frequencies, being the *Gli-B1m*/*Glu-B3i* the most frequents with 35 lines (Table 3).

Additional characterization of the diversity for the *Glu-A1*, *Glu-B1*, *Glu-D1*, *Glu-A3*, *Gli-B1*/*Glu-B3* and *Glu-D3* loci is presented in Table 4. The average total genetic diversity (*Ht*) across the six loci in the seven provinces was equal to 0.613 (ranging from 0.308 for *Glu-D1* to 0.783 for *Glu-A3* and *Gli-B1*/*Glu-B3*). This diversity can be divided into two components, *Hs* and *Dst* (Table 4), measuring the genetic diversity within and among provinces (Nei 1973). The relative average differentiation among provinces was *Gst* = 15.45% (ranging from 10.48% for *Glu-B1* to 20.04% for *Glu-A3*).

**Table 4.** Genetic diversity within and between origins in the evaluated accessions

| Locus | Alleles | *Ht* | *Hs* | *Dst* | *Gst* (%) |
|---|---|---|---|---|---|
| *Glu-A1* | 4 | 0.562 | 0.467 | 0.094 | 16.77 |
| *Glu-B1* | 7 | 0.635 | 0.569 | 0.067 | 10.48 |
| *Glu-D1* | 4 | 0.308 | 0.270 | 0.038 | 12.32 |
| *Glu-A3* | 6 | 0.783 | 0.626 | 0.157 | 20.04 |
| *Gli-B1*/*Glu-B3* | 11 | 0.783 | 0.648 | 0.135 | 17.28 |
| *Glu-D3* | 3 | 0.607 | 0.529 | 0.077 | 12.76 |
| Mean | | 0.613 | 0.518 | 0.095 | 15.45 |

*Ht* total gene diversity; *Hs* average gene diversity within origins; *Dst* average gene diversity between origins; and *Gst* gene diversity between origins, relative to *Ht*

## Discussion

Wheat has been cultivated since ancient times in Andalusia, which under Roman rule was called Baetica. Agricultural systems were transformed in Spain after the 1960s and mechanized harvesting was introduced requiring changes in the cultivars used by farmers. In the case of wheat, the traditional

varieties were substituted by modern ones with higher yields and adapted to the mechanical harvest due to their lower plant height. Although some landraces are still cultivated in marginal areas of this vast region, mainly in the northern area of Granada province, most of the old wheat landraces are only conserved nowadays in Genebanks, although information on them according with their passport data is often scarce. Flour from these old varieties or landraces could gain importance with the revival of interest in traditional products as the enclosed ones inside of the PGI 'Pan de Alfacar', which were made with it. Of particular importance is that the heterogeneity of these landraces could be in danger due to the normal handling (regeneration and storage) of these materials in Genebanks, according to several studies (Steiner et al. 1997; Börner et al. 2000).

In this study, old Andalusian landraces obtained from two Genebanks were evaluated. Two main handicaps of theses landraces include difficulties cultivating them using modern farm equipment due to their height and their lower grain yield compared to modern cultivars. However, the properties of the flour they produce are more desirable than that produced from modern wheat for making specific traditional products of the region. The future use of these materials is dependent upon an increase in demand for traditional products, a developing trend in western countries. Additionally, these old varieties could serve as models for the development of new wheat cultivars with such modern agronomical traits as short height, high yield, disease resistance, but that produce the same type of flour.

The materials analysed in the current study were classified into three different sets according to their variation: accessions with homogeneity for both morphological traits and for glutenins composition; accessions homogeneous for morphological traits but heterogeneous for glutenins composition; and accessions heterogeneous for both ones.

The morphological analysis performed indicated that of the 22 that Lagasca and Rojas Clemente enclosed in the «*Ceres Hispanica*» herbarium for common wheat (Téllez Molina and Peña 1952) only five botanical varieties were found in this set of materials and represent a small portion of old Spanish wheat. It is important to emphasise that the conservation of cultivated material is largely determined by the practical needs of farmers and what they are planting at the moment of collection and, consequently, it is very difficult to establish how much genetic diversity could have been missed during scientists' collection missions. Keeping this in mind, Ruiz et al. (2002) found 10 botanical varieties in a collection of 59 common wheat landraces from other Spanish regions – their most frequent finding was the white grain var. *graecum*. Similar results were found during analysis of the Mexican Creole wheat landraces (Caballero et al. 2010), in which seven botanical varieties were found and var. *graecum* was also the most frequent; whereas in the Andalusian materials analysed, the most frequent botanical variety was the red grain var. *aestivum*.

The variation observed for the glutenin composition is more difficult to associate specifically with farmers' needs, although it could be related to their knowledge of how bakers used these landraces and the properties required to make good bread, biscuits, porridge and other products. The traditional breads from some areas in Andalusia are generally baked using flour with medium/weak gluten strength, which could justify the high frequency of the HMWGs combinations with low *Glu-1* quality score.

With respect to the HMWG composition, the study by Ruiz et al. (2002) showed three, five and four alleles for the *Glu-A1*, *Glu-B1* and *Glu-D1* loci, respectively. These same alleles were also detected by (Giraldo et al. 2010), who analysed 169 common wheat landraces, although in this last study two new alleles appeared, one for the *Glu-A1* (subunit 2‥) and other one for the *Glu-B1* (subunits 7*+9). None of these new alleles were found in the

Andalusian wheat, but two novel alleles (subunit 1'" for the *Glu-A1* locus or subunits 3*+T2 for the *Glu-D1* ones) were detected. The comparison of the Mexican wheat varieties showed clear differences in the frequencies of the HMWGs alleles, so for the *Glu-A1* locus the Mexican wheat presented the *Glu-A1a* and *Glu-A1c* alleles at 9 and 39%, respectively; in Andalusian wheat these percentages are inverse (*Glu-A1a*: 22% and *Glu-A1c*: 10%). Similar changes can be observed for the *Glu-B1* locus, where the *Glu-B1b* changed to 39% in Mexican to 11% in Andalusian wheat, or the *Glu-B1i* allele, 31% in Mexican and 4% in Andalusian ones. In both collections, the subunit 2+12 (*Glu-D1a*) associated with inferior bread-making quality was the most frequent for the *Glu-D1* locus, while the subunits 5+10 (good quality) appeared in only 8 to 9%. Among the LMWGs, *Glu-B3* had the highest diversity across all subpopulations (Macharia et al. 2014). Igrejas et al. (1999) also observed high diversity in the *Glu-B3* locus followed by the *Glu-A3* and *Glu-D3* loci in collection of 63 different kinds of Portuguese bread wheat. A circumstance common to all these studies is the presence of the hegemonic alleles together with others that should be classified as rare or very rare, which could be missing by processes of genetic drift.

Although further studies should be carried out, the characterization of these landraces could permit the evaluation of the relationship between the uses of these flours and their seed storage proteins composition. The knowledge of these characteristics may allow the design of new cultivars with quality traits desired for the elaboration of traditional foods. The data presented is also very useful for the recovery of the old varieties or landraces by farmers interested in planting those materials – such grain could have an added value in the market.

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## Supplementary

Table S1: **Supplementary Table**

| UCO | NUMBAN | NOMLOC | Locality | Plant: length | Section of stem | Apical rachis segment: hairiness of convex surface | Glumes colour | Glumes hairiness | Awn or scurs: presence | Ear: shape in profile | Ear: density | Lower glume: shoulder width | Lower glume: shoulder shape | Grain colour | *Glu-A1* | *Glu-B1* | *Glu-D1* | *Glu-A3* | *Glu-B1/ Glu-B3* | *Glu-D3* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | BGE013207 | Sierra Nevada-A | Granada | very long | thin | null | white | glabrous | present | fusiform | medium | narrow | slightly | red | 1 | 7+null | 2+12 | f | h/d | a |
| 2 | BGE008216 | Trigo de Almería-A | Almería | medium | medium | null | white | glabrous | present | fusiform | very dense | absent | sloping | white | 2* | 17+18 | 2+12 | b | c/g | b |
| | BGE008216 | Trigo de Almería-A | Almería | medium | medium | null | white | glabrous | present | fusiform | very dense | absent | sloping | white | 2* | 17+18 | 2+12 | c | c/g | b |
| 4 | BGE008218 | Trigo de Almería-B | Almería | very long | medium | null | white | glabrous | present | fusiform | dense | absent | sloping | white | 2* | 20t+20y | 2+12 | f | m/i* | b |
| | BGE008218 | Trigo de Almería-B | Almería | very long | medium | null | white | glabrous | present | fusiform | dense | absent | sloping | white | 2* | 20t+20y | 2+12 | f | m/i | c |
| 5 | BGE011877 | Desconocido de Almería | Almería | very long | thin | null | white | glabrous | absent | fusiform | dense | medium | straight | white | 2* | 7+9 | 5+10 | g | i/c' | b |
| 6 | BGE011987 | Candeal compacto | Almería | very long | medium | null | white | glabrous | present | claviform | very dense | narrow | slightly | red | 1 | 20t+20y | 2+12 | d | h/d* | b |
| | BGE011987 | Candeal compacto | Almería | very long | medium | null | white | glabrous | present | claviform | very dense | narrow | slightly | red | 1 | 20t+20y | 2+12 | d | h/d* | c |
| 7 | BGE012113 | Candeal mocho | Almería | very long | thin | null | white | glabrous | absent | fusiform | medium | medium | straight | white | 2** | 20t+20y | 3+12 | f | * | b |
| 9 | BGE013156 | Rojo | Almería | very long | thin | medium | white | glabrous | present | fusiform | dense | medium | straight | red | 2* | 20t+20y | 2+12 | f | m/i* | a |
| 10 | BGE023727 | Desconocido de Vélez Rubio | Almería | very long | medium | null | white | glabrous | present | fusiform | medium | medium | elevated | red | 2* | 6+8 | 2+12 | d | i/c' | b |
| | BGE023727 | Desconocido de Vélez Rubio | Almería | very long | medium | null | white | glabrous | present | fusiform | medium | medium | elevated | red | 1 | 20t+20y | 2+12 | g | h/d* | b |
| 12 | BGE026946 | Pichi-Las tres Villas | Almería | very long | medium | null | white | glabrous | absent | fusiform | dense | medium | straight | white | 2* | 20t+20y | 3+12 | f | m/i* | b |
| | BGE026946 | Pichi-Las tres Villas | Almería | very long | medium | null | white | glabrous | absent | fusiform | dense | medium | straight | red | 2* | 20t+20y | 5+10 | c | m/i | b |
| | BGE026946 | Pichi-Las tres Villas | Almería | very long | medium | null | white | glabrous | absent | fusiform | dense | medium | straight | red | 2* | 20t+20y | 3+12 | e | m/i | a |
| 13 | BGE026947 | Pichi-Chirivel | Almería | very long | medium | null | white | glabrous | absent | fusiform | medium | medium | straight | white | 2* | 20t+20y | 3+12 | f | m/i* | b |
| | BGE026947 | Pichi-Chirivel | Almería | very long | medium | null | white | glabrous | absent | fusiform | medium | medium | straight | white | 1 | 20t+20y | 2+12 | d | b/b | a |
| | BGE026947 | Pichi-Chirivel | Almería | very long | medium | null | white | glabrous | absent | fusiform | medium | medium | straight | white | 2* | 20t+20y | 2+12 | f | f/g | b |
| | BGE026947 | Pichi-Chirivel | Almería | very long | medium | null | white | glabrous | absent | fusiform | medium | medium | straight | red | 1 | 20t+20y | 2+12 | d | m/i* | b |
| 14 | BGE026948 | Pichi-Gergal | Almería | very long | medium | null | white | glabrous | absent | fusiform | medium | medium | slightly | white | 2* | 7+8 | 2+12 | f | e/f | a |
| | BGE026949 | Pichi-Alcontar | Almería | very long | medium | null | white | glabrous | absent | fusiform | medium | medium | slightly | red | 2** | 20t+20y | 2+12 | f | m/i | c |
| 15 | BGE026949 | Pichi-Alcontar | Almería | very long | medium | null | white | glabrous | absent | fusiform | medium | narrow | slightly | red | 2* | 20t+20y | 5+10 | c | q/b* | b |
| | BGE026949 | Pichi-Alcontar | Almería | very long | medium | null | white | glabrous | absent | fusiform | medium | narrow | slightly | white | 2* | 20t+20y | 3+12 | f | m/i | b |
| | BGE026949 | Pichi-Alcontar | Almería | very long | medium | null | white | glabrous | absent | fusiform | medium | narrow | slightly | white | 2* | 20t+20y | 2+12 | f | h/d* | b |
| 17 | BGE030918 | Pichi-Senes | Almería | long | thin | medium | white | glabrous | absent | fusiform | very dense | broad | straight | red | null | 7+null | 5+10 | d | b/b' | b |
| | BGE030918 | Pichi-Senes | Almería | long | thin | medium | white | glabrous | absent | fusiform | very dense | broad | straight | red | null | 7+null | 5+10 | d | b/b | b |
| 21 | BGE013163 | Barbilla-A | Cádiz | very long | thin | null | white | pubescent | present | fusiform | laxa | narrow | slightly | red | 2** | 13+16 | 2+12 | g | g/f | b |
| 22 | BGE020363 | Trigo de Grazalema | Cádiz | very long | thin | medium | white | glabrous | present | fusiform | medium | narrow | slightly | white | 1 | 7+8 | 5+10 | f | h/d* | b |
| | BGE020363 | Trigo de Grazalema | Cádiz | very long | thin | medium | white | glabrous | present | fusiform | medium | narrow | slightly | red | 2* | 20t+20y | 3+12 | f | c/g* | b |
| 23 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |

| UCO | NUMBAN | NOMLOC | Locality | Plant: length | Section of stem | Apical rachis segment: hairiness of convex surface | Glumes colour | Glumes hairiness | Awn or seurs: presence | Ear: shape in profile | Ear: density | Lower glume: shoulder width | Lower glume: shoulder shape | Grain colour | *Glu-A1* | *Glu-B1* | *Glu-D1* | *Glu-A3* | *Glu-B1/ Glu-B3* | *Glu-D3* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 24 | BGE008213 | Blanquillo | Córdoba | very long | medium | null | white | glabrous | present | fusiform | dense | narrow | slightly | white | 2* | 20x+20y | 2+12 | f | h/d | c |
| | BGE008213 | Blanquillo | Córdoba | very long | medium | null | white | glabrous | present | fusiform | dense | narrow | slightly | white | 2* | 20x+20y | 2+12 | e | h/d | c |
| | BGE008213 | Blanquillo | Córdoba | very long | medium | null | white | glabrous | present | fusiform | dense | narrow | slightly | white | 2* | 20x+20y | 2+12 | d | h/d | c |
| 25 | BGE008214 | Trigo de Córdoba-B | Córdoba | very long | medium | null | white | glabrous | present | fusiform | medium | narrow | slightly | white | 2* | 20x+20y | 2+12 | f | m/i* | b |
| | BGE008214 | Trigo de Córdoba-B | Córdoba | very long | medium | null | white | glabrous | present | fusiform | medium | narrow | slightly | red | 2* | 20x+20y | 2+12 | g | m/i* | b |
| 26 | BGE011825 | Ratón de Belalcazar | Córdoba | long | medium | medium | white | glabrous | absent | fusiform | laxa | narrow | slightly | white | 1 | 7+8 | 2+12 | f | f/g | c |
| 27 | BGE011829 | Rabón de Hinojosa | Córdoba | very long | thin | medium | white | glabrous | absent | fusiform | laxa | narrow | slightly | white | 1 | 7+9 | 2+12 | f | b/b | b |
| 30 | BGE008245 | Trigo de Granada-A | Granada | long | thin | null | white | glabrous | absent | fusiform | very dense | medium | straight | white | null | 7+8 | 2+12 | c | m/i | c |
| | BGE008245 | Trigo de Granada-A | Granada | long | thin | null | white | glabrous | absent | fusiform | very dense | medium | straight | red | null | 13+16 | 2+12 | c | m/i | b |
| 31 | BGE012127 | Candeal de Sierra Nevada | Granada | very long | medium | null | white | glabrous | absent | fusiform | medium | medium | straight | white | 2* | 20x+20y | 2+12 | d | m/i | b |
| 32 | BGE015384 | Trigo del Gobierno-A | Granada | very long | medium | null | white | pubescent | present | fusiform | very dense | narrow | slightly | red | 2* | 20x+20y | 2+12 | e* | h/d | a |
| 33 | BGE015390 | Trigo de Granada-B | Granada | very long | medium | null | white | glabrous | present | fusiform | medium | medium | straight | red | 2* | 20x+20y | 2+12 | e* | h/d | c |
| | BGE015390 | Trigo de Granada-B | Granada | very long | medium | null | white | glabrous | present | fusiform | medium | medium | straight | white | 2* | 20x+20y | 2+null | f | m/i* | b |
| | BGE015390 | Trigo de Granada-B | Granada | very long | medium | null | white | glabrous | present | fusiform | medium | medium | straight | red | null | 20x+20y | 2+12 | e* | h/d | c |
| 35 | BGE017171 | Mocho | Granada | long | thin | null | white | glabrous | present | fusiform | very dense | medium | straight | white | 2* | 7+8 | 2+12 | c | m/i | b |
| 36 | BGE018246 | Sierra Nevada cañihueca | Granada | very long | thin | null | white | glabrous | present | fusiform | medium | narrow | slightly | red | 1 | 7+null | 2+12 | f | h/d | a |
| 37 | BGE018254 | Sierra Nevada-B | Granada | very long | thin | null | white | glabrous | present | fusiform | dense | medium | straight | red | 1 | 7+null | 2+12 | f | h/d | a |
| 38 | BGE018668 | Desconocido de Puebla de Don Fadrique | Granada | long | medium | null | white | glabrous | present | fusiform | dense | medium | straight | red | 2* | 20x+20y | 2+12 | f | m/i* | c |
| | BGE018668 | Desconocido de Puebla de Don Fadrique | Granada | long | medium | null | white | glabrous | present | fusiform | dense | medium | straight | red | 2* | 20x+20y | 3+12 | f | m/i* | c |
| 40 | BGE018670 | Trigo de Soportujar | Granada | long | thin | null | white | glabrous | absent | fusiform | very dense | broad | straight | white | null | 7+8 | 2+12 | c | m/i | c |
| | BGE018670 | Trigo de Soportujar | Granada | long | thin | null | white | glabrous | absent | fusiform | very dense | broad | straight | red | null | 20x+20y | 2+12 | c | m/i* | c |
| | BGE018670 | Trigo de Soportujar | Granada | long | thin | null | white | glabrous | absent | fusiform | very dense | broad | straight | red | null | 20x+20y | 2+12 | c | m/i* | a |
| | BGE018670 | Trigo de Soportujar | Granada | long | thin | null | white | glabrous | absent | fusiform | very dense | broad | straight | red | null | 7+8 | 2+12 | c | m/i* | a |
| 41 | BGE020362 | Candeal | Granada | very long | thin | null | white | glabrous | absent | fusiform | laxa | narrow | slightly | white | 2* | 20x+20y | 3+12 | f | m/i* | b |
| | BGE020362 | Candeal | Granada | very long | thin | null | white | glabrous | absent | fusiform | laxa | narrow | slightly | white | 2* | 20x+20y | 2+12 | d | m/i | b |
| 43 | BGE029101 | Trigo Granada 24 | Granada | very long | thin | null | white | glabrous | absent | fusiform | medium | medium | straight | white | 2* | 20x+20y | 2+12 | d | m/i* | b |
| | BGE029101 | Trigo Granada 24 | Granada | very long | thin | null | white | glabrous | absent | fusiform | medium | medium | straight | red | 2* | 20x+20y | 2+12 | e* | m/i* | a |
| 44 | BGE030917 | Trigo del Gobierno-B | Granada | very long | medium | null | white | glabrous | absent | fusiform | dense | medium | elevated | red | 2* | 20x+20y | 2+12 | f | m/i | a |
| | BGE030917 | Trigo del Gobierno-B | Granada | very long | medium | null | white | glabrous | absent | fusiform | dense | medium | elevated | red | 2* | 20x+20y | 2+12 | e | e/f | b |
| 45 | BGE011889 | Rojo pelón | Huelva | very long | medium | null | white | glabrous | absent | fusiform | dense | narrow | slightly | red | 2* | 20x+20y | 2+12 | c | q/b | a |

| UCO | NUMBAN | NOMLOC | Locality | Plant: length | Section of stem | Apical rachis segment: hairiness of convex surface | Glumes colour | Glumes hairiness | Awn or scurs: presence | Ear: shape in profile | Ear: density | Lower glume: shoulder width | Lower glume: shoulder shape | Grain colour | *Glu-A1* | *Glu-B1* | *Glu-D1* | *Glu-A3* | *Glu-B1/ Glu-B3* | *Glu-D3* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 46 | BGE012196 | Pelón blanco | Huelva | very long | medium | medium | white | glabrous | absent | fusiform | dense | medium | straight | red | 2** | 13+16 | 2+12 | b | g/f | a |
| 47 | BGE013154 | Barbilla blanca | Huelva | very long | medium | null | white | glabrous | present | fusiform | medium | narrow | slightly | red | 2* | 20x+20y | 2+12 | f | m/i* | b |
| | BGE013154 | Barbilla blanca | Huelva | very long | medium | null | white | glabrous | present | fusiform | medium | narrow | slightly | red | 2* | 20x+20y | 2+12 | f | m/i* | c |
| 48 | BGE018234 | Barbilla roja | Huelva | very long | medium | medium | white | glabrous | present | fusiform | medium | narrow | slightly | red | 1 | 13+16 | 2+12 | f | q/b | a |
| | BGE018234 | Barbilla roja | Huelva | very long | medium | medium | white | glabrous | present | fusiform | medium | narrow | slightly | red | 2* | 13+16 | 2+12 | f | q/b | a |
| 50 | BGE036374 | Trigo barbilla rerbero | Huelva | very long | medium | null | white | glabrous | present | fusiform | dense | medium | straight | red | 2* | 7+null | 2*+T2* | e* | g/f | a |
| | BGE036374 | Trigo barbilla rerbero | Huelva | very long | medium | null | white | glabrous | present | fusiform | dense | medium | straight | red | 2* | 13+16 | 2+12 | e* | m/i | b |
| | BGE036374 | Trigo barbilla rerbero | Huelva | very long | medium | null | white | glabrous | present | fusiform | dense | medium | straight | red | 2* | 13+16 | 2+12 | e | m/i | a |
| | BGE036374 | Trigo barbilla rerbero | Huelva | very long | medium | null | white | glabrous | present | fusiform | dense | medium | straight | red | 1 | 13+16 | 2+12 | b | c/g* | a |
| 51 | BGE008215 | Trigo de Jaén | Jaén | medium | medium | medium | white | glabrous | present | fusiform | dense | medium | straight | white | 2* | 17+18 | 2+12 | c | c/g* | b |
| 54 | BGE008212 | Trigo de Malaga | Malaga | very long | medium | medium | white | glabrous | absent | fusiform | dense | medium | straight | red | 2* | 7+8 | 2+12 | g | f/g | b |
| 55 | BGE019331 | Desconocido de Málaga-A | Málaga | very long | thin | null | white | glabrous | absent | fusiform | laxa | medium | straight | red | 2* | 7+8 | 2+12 | g | f/g | b |
| 56 | BGE019332 | Desconocido de Málaga-B | Malaga | very long | thin | null | white | glabrous | present | fusiform | medium | narrow | slightly | red | 2* | 20x+20y | 2+12 | f | f/g | c |
| | BGE019332 | Desconocido de Málaga-B | Malaga | very long | thin | null | white | glabrous | present | fusiform | medium | narrow | slightly | red | 1 | 7+null | 2+12 | e | q/b | a |
| 61 | PI-191017 | Barbilla de Cádiz | Cádiz | very long | medium | medium | white | glabrous | present | fusiform | very dense | medium | straight | red | 2* | 20x+20y | 2+12 | f | c/g* | a |
| 62 | PI-191018 | Barbilla de Huelva | Huelva | very long | medium | medium | white | glabrous | present | fusiform | dense | medium | straight | red | 2* | 13+16 | 2+12 | f | q/b | a |
| 81 | PI-191046 | Candeal de Granada | Granada | very long | thin | null | white | glabrous | absent | fusiform | medium | medium | straight | white | 1 | 20x+20y | 3+12 | d | b/b | b |
| 194 | PI-191215 | Sierra Nevada-B | Granada | very long | thin | null | white | glabrous | present | fusiform | dense | medium | straight | red | 1 | 7+null | 2+12 | f | m/i | a |
| 244 | PI-191431 | Mocho Blanco del Valle | Granada | very long | thin | medium | white | glabrous | absent | fusiform | laxa | narrow | slightly | white | 1 | 7+null | 2+12 | d | m/i | a |
| 422 | PI-136567 | Barbilla-B | Cádiz | very long | thin | null | white | glabrous | present | fusiform | medium | medium | slightly | red | 2* | 20x+20y | 2+12 | f | m/i* | a |

## CAPÍTULO IV

# CARACTERIZACIÓN MOLECULAR DE ALELOS WAXY EN TRES SUBESPECIES DE TRIGOS HEXAPLOIDES E IDENTIFICACIÓN DE DOS NUEVOS ALELOS *WX-B1*

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**Resumen**

La composición del almidón del grano de trigo tiene una influencia primordial en la calidad de la harina. El almidón se compone de dos tipos de polímeros de glucosa: amilosa (22-35% del total) y amilopectina (68-75% del total). La amilosa es sintetizada por las proteínas waxy. Varios estudios han contribuido al catálogo de alelos waxy disponibles para los mejoradores, aunque sigue la búsqueda de nuevos alelos de estas y otras proteínas relacionadas con la calidad de la harina. En este trabajo se describe la caracterización de dos alelos nuevos *Wx-B1* (*Wx-B1k* y *Wx-B1m*) en una colección de trigo macha, trigo enano de la India y trigo cabezorro o club. Se detectaron que varias accesiones carecen de la proteína *Wx-B1*, siendo esto causado en algunos casos por el alelo nulo común *Wx-B1b*. De las otras accesiones, cuatro trigos enanos de la India mostraron la inserción de 4 pb en el séptimo exón, y una de trigo cabezorro o club tenía una deleción de cuatro nucleótidos en el segundo exón. Estas mutaciones eran nuevas y catalogadas provisionalmente como *Wx-B1k* y *Wx-B1m*, respectivamente, y podrían utilizarse para ampliar la variabilidad genética de este gen.

**Palabras clave:** proteínas waxy; genes waxy; calidad del trigo; almidón de trigo; alelos *nulos*.

**Abstract**

The starch composition of wheat grain has a primary influence on flour quality. Wheat starch consists of two types of glucose polymers: amylose (22-35% of the total) and amylopectin (68-75% of the total). Amylose is synthesized by waxy proteins. Several studies have contributed to the catalogue of *waxy* alleles available for breeders, and the search for novel alleles of these and other proteins related to flour quality continues. In this report, we describe the characterization of two novel *Wx-B1* alleles (*Wx-B1k* and *Wx-B1m*) in a collection of macha, Indian dwarf and club wheat. Several accessions lacking Wx-B1 protein were detected, and some were caused by the common *Wx-B1b null* allele. Of the other accessions, four from of Indian dwarf wheat showed the insertion of 4-bp within the seventh exon, and one from of club wheat had a deletion of four nucleotides in the second exon. These mutations were novel and provisionally catalogued as *Wx-B1k* and *Wx-B1m*, respectively, and could be used to enlarge the genetic variability for this gene.

**Keywords:** waxy proteins; waxy genes; wheat quality; wheat starch; *null* alleles.

**Introduction**

To define the processing and end-use quality of wheat, grain hardness and gluten properties have often been considered the most important factors. They are responsible for two of the most important properties of wheat dough: its water absorption capacity and its visco-elastic properties. However, the increase in demand for novel, high-quality processed food has focussed attention on other components of the grain that also have an important effect on wheat industrial quality. One example is starch (Rahman et al. 2000), the main component of wheat grain, representing 65-75% of its dry weight.

Starch is formed by two glucose polymers: linear amylose (22-35% of the total) and highly branched amylopectin (68-75% of the total) (James et al. 2003). Because the amylose/amylopectin ratio affects such processing properties of wheat as gelatinization, pasting and gelation (Zeng et al. 1997), it is reasonable to expect that this could affect end-use quality of different wheat products such as bread, pasta and noodles (Martin et al. 2008; Miura and Tanii 1994; Park and Baik 2007) as well their shelf-life (Hayakawa et al. 2004) and nutritional value (Regina et al. 2006).

In recent decades, the role of different enzymes controlling starch composition/properties have been examined (Morell et al. 2001). Different starch synthases (SSI, SSII and SSIII) have been shown to function together with the branching (SBEI and SBEII) and debranching enzymes (SDBEs) in the synthesis of amylopectin, while the Granule-Bound Starch Synthase I (GBSSI), or waxy protein is the sole enzyme responsible for amylose synthesis. In common wheat (*Triticum aestivum* L. ssp. *aestivum*; 2n = 6× = 42, BBAADD), one waxy protein for each genome has been detected - these Wx proteins are encoded by genes *Wx-A1*, *Wx-B1* and *Wx-D1* located on

chromosomes 7AS, 4AL (translocated from original 7BS) and 7DS, respectively (Yamamori et al. 1994). Although different studies on the variation of these enzymes have been carried out and several variants of starch (amylose and amylopectin) synthesis enzymes have been detected (Yamamori et al. 1994, 1995; Yamamori and Endo 1996), variability in modern wheat cultivars is not very wide according to data in the Wheat Gene Catalogue (McIntosh et al. 2013). However, the use of the null variants detected has permitted the development of wheat lines with novel starch properties (Nakamura et al. 1995, 2006; Yamamori 2009; Yamamori and Quynh 2000; Yamamori and Yamamoto 2011), which have shown remarkable differences in terms of industrial (Graybosch 1998) and nutritional quality (Yamamori et al. 2006).

Species in the primary wheat gene pool could be good candidates as gene sources for the search of novel Wx variants to enable diversification of the starch properties of wheat. Within the hexaploid species of this gene pool, there are some neglected or underutilized subspecies, such as club, Indian dwarf or macha wheat, which have not been screened for waxy proteins variability. Club wheat [*T. aestivum* L. ssp. *compactum* (Host) Mackey], characterized by a compact spike, is distributed throughout the Old World (Filatenko and Hammer 2014), and has commercial importance in the US Pacific Northwest area for production of flours suitable for making cookies. The distribution areas of the other two subspecies are more limited. Indian dwarf wheat [*T. aestivum* L. ssp. *sphaerococcum* (Percival) Mackey] has small stature and small round grains, and originated in India and Pakistan (Hosono 1954). Macha wheat [*T. aestivum* L. ssp. *macha* (Dekapr. & A.M. Menabde) Mackey] is a hulled wheat endemic to the Caucasus area. An important advantage of these three cultivated subspecies is that they cross readily with modern wheat and have little linkage drag of unwanted traits. Until now, several studies using Indian dwarf wheat have shown different interesting

traits for genetic improvement, e.g. rust resistance (Chen et al. 2012), salt tolerance (Badridze et al. 2009), concentration of bioactive compounds in grain (Giambanelli et al. 2013) and even high grain yield (Zwer et al. 1995). Consequently, these wheat subspecies could be used to widen the genetic diversity of modern wheat for waxy proteins and other agronomic traits.

The aim of the present study was to evaluate the variability for waxy proteins in a collection of club, Indian dwarf and macha wheat, together with the molecular characterization of the polymorphic *waxy* alleles found.

## Material and methods

*Plant material*

Forty-three accessions of club wheat, twenty-seven accessions of macha wheat, and twenty-three accessions of Indian dwarf wheat obtained from the National Small Grain Collections (Aberdeen, USA) were analysed in this study (Electronic Supplementary Material, ESM-1). The bread wheat cvs. Chinese Spring and Kanto 107 were used as standards.

*Starch extraction and electrophoretic analysis*

Whole grain flour from a single grain was mixed with 1 ml of distilled water and incubated at 4ºC for 24 h. The homogenate was filtered through Miracloth and centrifuged at 14000 g for 1.5 min. The pellet was washed with 1 ml of buffer A [55 mM Tris-HCl pH 6.8, 2.3% (w/v) sodium dodecyl sulphate, 2% (w/v) dithiotreitol, 10% (v/v) glycerol], according to Echt and Schwartz (1981). Then 1 ml of buffer A was added to the pellet and left for 30 min at room temperature. The pellet was washed three times with distilled water, once with acetone and then air-dried. The residue was mixed with 80 µl of buffer A containing 0.02% (w/v) bromophenol blue, heated in a boiling bath for 2 min, cooled in ice and centrifuged.

Aliquots of supernatant (20 µl) were loaded in vertical SDS-PAGE slabs in a discontinuous Tris-HCl-SDS buffer system (pH: 6.8/8.8) at a polyacrylamide concentration of 12% (w/v, C: 0.44%). The Tris-HCl/glycine buffer system of Laemmli (1970) was used. Electrophoresis was performed at a constant current of 30 mA/gel and 18ºC, continuing for 4 hours after the tracking dye migrated off the gel. Protein bands were visualised by silver staining.

For two-dimensional polyacrylamide-gel electrophoresis (2D-PAGE), starch purified as described above was incubated at room temperature in 300 µl of lysis buffer [8 M urea, 2% Triton X-100, 2% ampholine pH 3.5-10 (Pharmacia LKB) and 5% 2-mercaptoethanol]. After centrifugation, the supernatant containing the solubilised proteins was subjected to 2D-PAGE using isoelectric focusing (IEF) for the first dimension and modified SDS-PAGE for the second (Nakamura et al. 1993a). IEF gels contained 2.5% (v/v) ampholines (pH 3.5-10/5-8, 1:1). Focusing was begun from the acidic end (0.01 M $H_3PO_4$) and continued at 400 V for 15 h, then 800 V for 60 min at room temperature. Proteins were revealed by silver staining according to Silver stain kit (Wako Pure Chemical Industries, Ltd., Japan).

*DNA extraction and PCR amplification*

For DNA extraction, approximately 100 mg of young leaf tissue was excised, immediately frozen in liquid nitrogen and stored at -80°C. Genomic DNA was extracted by the CTAB method (Stacey and Isaac 1994).

The genomic sequence of the *Wx* gene contains twelve exons and eleven introns, with a coding region around 2,800 bp. Primers designed by Guzmán and Alvarez (2012) were used to amplify the coding region of *Wx* genes in three regions or fragments: the first from second to fourth exon (Wx1Fw/1Rv); the second from fourth to the seventh exon (Wx2Fw/2Rv); and the last fragment from the seventh to the twelfth exon (Wx3Fw/3Rv).

All amplifications were performed in 20 µl of final reaction volume containing 50 ng of DNA genomic, 1.25 mM $MgCl_2$, 0.2 mM dNTPs, 4µl 10× PCR buffer and 0.75 U Taq polymerase (Promega, Madison, WI, USA). The primers concentrations were 0.4, 0.3 and 0.2 µM of each primer for the first, second, and third fragment respectively. Furthermore, the primers BDFL and BRD designed by Nakamura et al. (2002) were used to detect *Wx-B1b* allele following the author´s instructions. Also, a new reverse primer (Wx1.3Rv) was designed to amplify the beginning region of these genes. PCR conditions as well as primers sequence are available in Table 1.

**Table 1.** Description of PCR primers pairs for amplifying

| | | | | |
|---|---|---|---|---|
| Primers designed by Guzmán and Alvarez (2012) | | | | |
| Wx1 | Fw: 5′-TTGCTGCAGGTAGCCACACC-3′ | | | |
| | Rv: 5′-CCGCGCTTGTAGCAGTGGAA-3′ | | | |
| Wx2 | Fw: 5′-ATGGTCATCTCCCCGCGCTA-3′ | | | |
| | Rv: 5′-GTTGACGGCGAGGAACTTGT-3′ | | | |
| Wx3 | Fw: 5′-GGCATCGTCAACGGCATGGA-3′ | | | |
| | Rv: 5′-TTCTCTCTTCAGGGAGCGGC-3′ | | | |
| Primers designed by Nakamura et al. (2002) | | | | |
| BDFL | 5′-CTGGCCTGCTACCTCAAGAGCAACT-3′ | | | |
| BRD | 5′-CTGACGTCCATGCCGTTGACGA-3′ | | | |
| Primer designed in this study | | | | |
| Wx1.3Rv | 5′-TAGCGCGGGGAGATGACCAT-3′ | | | |
| PCR conditions | | | | |
| Initial denaturation = 3 min at 94 °C | | | | |
| | Pair | Denaturation | Annealing | Extension |
| 35 cycles | Wx1 [Fw/Rv] | 40 s at 94 °C | 30 s at 64 °C | 1 min at 72 °C |
| | Wx2 [Fw/Rv] | 30 s at 94 °C | 30 s at 66 °C | 1 min 30 s at 72 °C |
| | Wx3 [Fw/Rv] | 40 s at 94 °C | 30 s at 64 °C | 1 min 30 s at 72 °C |
| | BDFL / BRD | 30 s at 94 °C | 30 s at 65 °C | 2 min at 72 °C |
| | Wx1Fx/Wx1.3Rv | 30 s at 94 °C | 20 s at 66 °C | 20 s at 72 °C |
| Final extension = 5 min at 72 °C | | | | |

*Analysis of PCR products, cloning and sequencing analysis*

Amplification products were fractionated in vertical PAGE gels at 8% (w/v C: 1.28%), and the bands were visualized by ethidium bromide staining. PCR products were purified using Sureclean Plus (Bioline) and cloned into pGEM T-easy vector (Promega) for sequencing. *Wx-B1* inserts were selected from the mix of *Wx-1* inserts (*Wx-A1*, *Wx-B1* and *Wx-D1*) based on the size and digestion pattern with specific endonucleases of each insert. Inserts were sequenced from at least three different clones using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Carlsban, CA, USA). The sequences were analysed and compared to the sequences of cv. Chinese Spring available in the databases (*Wx-A1a*: AB019622, *Wx-B1a*: AB019623, and *Wx-D1a*: AB019624) using Geneious Pro ver. 5.0.4 software (Biomatters Ltd.).

**Results**

*Waxy protein polymorphism and PCR analysis*

The SDS-PAGE electrophoresis of waxy proteins did not detect polymorphism for Wx-A1 or Wx-D1 proteins in any of the three species. All samples showed two bands with the same mobility as those of cv. Chinese Spring (Fig. 1a). However, five accessions of club wheat and 13 of Indian dwarf wheat lacked the Wx-B1 protein (ESM-1). In contrast, all accessions of macha wheat showed a band similar to Wx-B1 protein of Chinese Spring. These results were confirmed by 2D-PAGE (Fig. 1b).

To identify the cause of the lack of Wx-B1 protein, PCR markers described by Nakamura et al. (2002) that permit identification of the *Wx-B1b* allele (the most common *Wx-B1* null allele) were used to screen these accessions. In general, accessions that produced the three waxy proteins had the three PCR product bands corresponding to each *Wx-1* gene (Fig. 2, lane 4). Compositions of the 18 accessions that did not show the wild composition are presented in Table 2. Thirteen of the accessions lacking Wx-

B1 protein did not show the corresponding *Wx-B1* band as did cv. Kanto 107 (Fig. 2, lane 8), a positive control for the *Wx-B1b* allele (Table 2). However, five of the accessions showed a PCR product corresponding to *Wx-B1*. The accession PI 442911 showed a band of the same apparent size as for Chinese Spring (Fig. 2, lane 3), and accessions CItr 4923, PI 272580, PI 330556 and PI 352499 showed a *Wx-B1* band with less electrophoretic mobility (Fig. 2, lane 5).

**Table 2.** *Wx-1* composition of the 18 accessions analyzed that does not present the wild composition (*Wx-A1a*, *Wx-B1a*, *Wx-D1a*).

| *Wx-A1* | *Wx-B1* | *Wx-D1* | N | Accession |
|---|---|---|---|---|
| *T. aestivum* ssp. *compactum* | | | | |
| *a* | *b* | *a* | 4 | PI 357307, PI 442912, PI 442913, PI 565431 |
| *a* | *m* | *a* | 1 | PI 442911 |
| *T. aestivum* ssp. *sphaerococcum* | | | | |
| *a* | *b* | *a* | 9 | CItr 4529, PI 115818, PI 182118, PI 272581, PI 277164, PI 282451, PI 282452, PI 324492, PI 352498 |
| *a* | *k* | *a* | 4 | CItr 4923, PI 272580, PI 330556, PI 352499 |

Wx-B1 *sequences analysis*

The *Wx-B1* genes of accessions that showed a PCR product different from the *Wx-B1b* allele were cloned and sequenced to identify the reason for the absence of the Wx-B1 protein. The sequences obtained were compared to the *Wx-B1a* allele of Chinese Spring. The four accessions of Indian dwarf wheat showed the same mutation, carrying the insertion of four nucleotides (GGTA) in the seventh exon at position 1437 from the start codon (Fig. 3). This insertion caused a frameshift mutation that generated a stop codon (TGA) within the ninth exon, which would make a truncated protein. Comparing this sequence with others available in NCBI GenBank by BLAST revealed that this mutation was novel, and therefore the allele was

provisionally named *Wx-B1k* (NCBI ID: KP726909), according to international nomenclature (McIntosh et al. 2013).

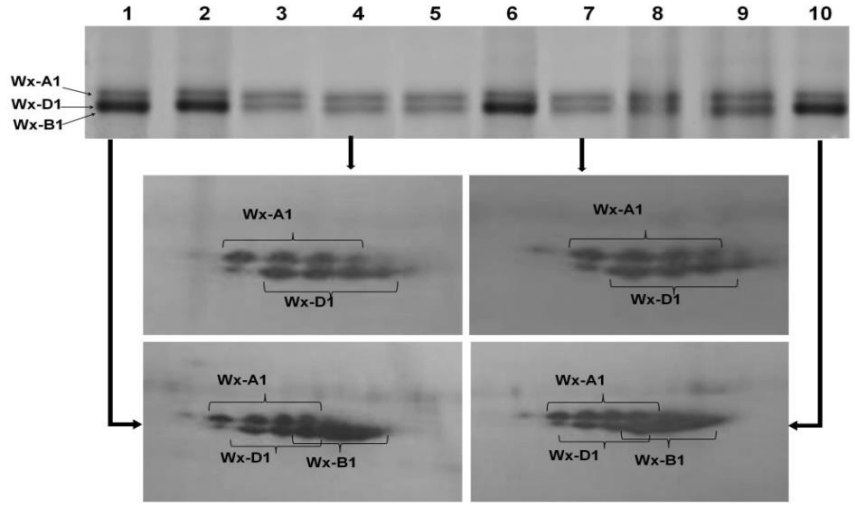


**Figure 1.** Waxy proteins polymorphism by SDS-PAGE and 2D electrophoresis means. All the samples had *Wx-A1* and *Wx-D1* proteins. *Lanes* are as follow: *1* PI 355512; *2* PI 422411; *3* PI 357307 (lack of *Wx-B1*); *4* PI 442911 (lack of *Wx-B1*); *5* PI 565431 (lack of *Wx-B1*); *6* cv. Chinese Spring; *7* PI 272580 (lack of *Wx-B1*); *8* PI 272581 (lack of *Wx-B1*); *9* PI 278650; and *10* CItr 4528.

In the case of accession PI 442911 (club wheat), its *Wx-B1* sequence (NCBI ID: KP726910) showed a deletion of four nucleotides (AACA) in the second exon (position 157 from the start codon) compared to the *Wx-B1a* allele (Fig. 4). This deletion caused a frame shift in the open reading frame (ORF) that translated in a stop codon 69-bp downstream. This mutation was also novel and provisionally catalogued as *Wx-B1m*.

*Development of a molecular marker to detect* Wx-B1m *allele*

Because the variation of the novel *Wx-B1m* allele was detected in the beginning of the gene, the sequence between positions -31 and 466 was amplified by the use of the Wx-1Fw primer together with a new reverse primer (Wx1.3Rv, Table 1). Three products of 492 bp (*Wx-B1a*), 480 bp

(*Wx-D1a*) and 472bp (*Wx-A1a*) were expected to be obtained in Chinese Spring by PCR with this primer pair. The PCR product was run in a polyacrylamide gel (Fig. 5, lanes 1-3). Chinese Spring (lane 1) showed only two bands, indicating that probably two of the *Wx-1* products (*Wx-A1* and *Wx-D1*) were co-migrating. This is in consonance with the profile of Kanto 107 (lane 3), which lacked the whole of *Wx-B1* gene, and its *Wx-A1b* gene is 23 nucleotides smaller than the *Wx-A1a* allele of Chinese Spring (Vrinten et al. 1999). In any case, the co-migration of both bands was confirmed by digesting the PCR product with the *Dde*I endonuclease, which had target sequences only in the *Wx-A1* product (lanes 4-6). The PCR digestion confirmed that the lower band of Chinese Spring (lane 1) and PI 442911 (lane 2) was composed by *Wx-A1* and *Wx-D1* products. Therefore, the upper band corresponded to *Wx-B1*, which presented two variants in addition to those of Kanto 107. In genotype PI 442911 (*Wx-B1m*), the *Wx-B1* band showed higher mobility than those of Chinese Spring (*Wx-B1a*), due to the deletion of four nucleotides as described above. Consequently, the use of this PCR assay permitted discrimination among *Wx-B1a*, *-B1b* and *-B1m* alleles

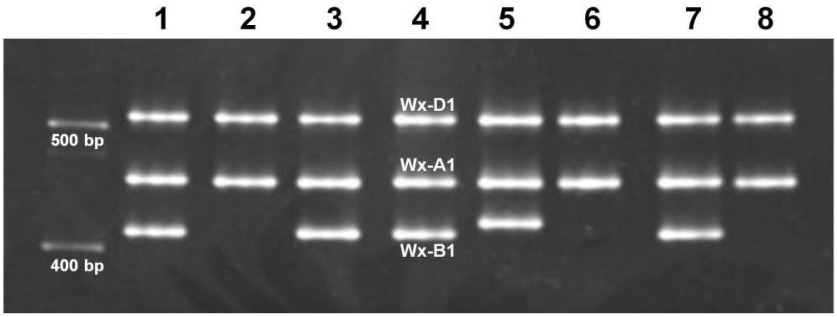


**Figure 2.** PCR analysis using primers BDFL/BRD from Nakamura et al. (2002). *Lanes* are as follow: *1* PI 422411; *2* PI 357307 (lack of *Wx-B1*); *3* PI 442911 (lack of *Wx-B1*); *4* cv. Chinese Spring; *5* PI 272580 (lack of *Wx-B1*); *6* PI 272581 (lack of *Wx-B1*); *7* PI 278650; and *8* Kanto 107.

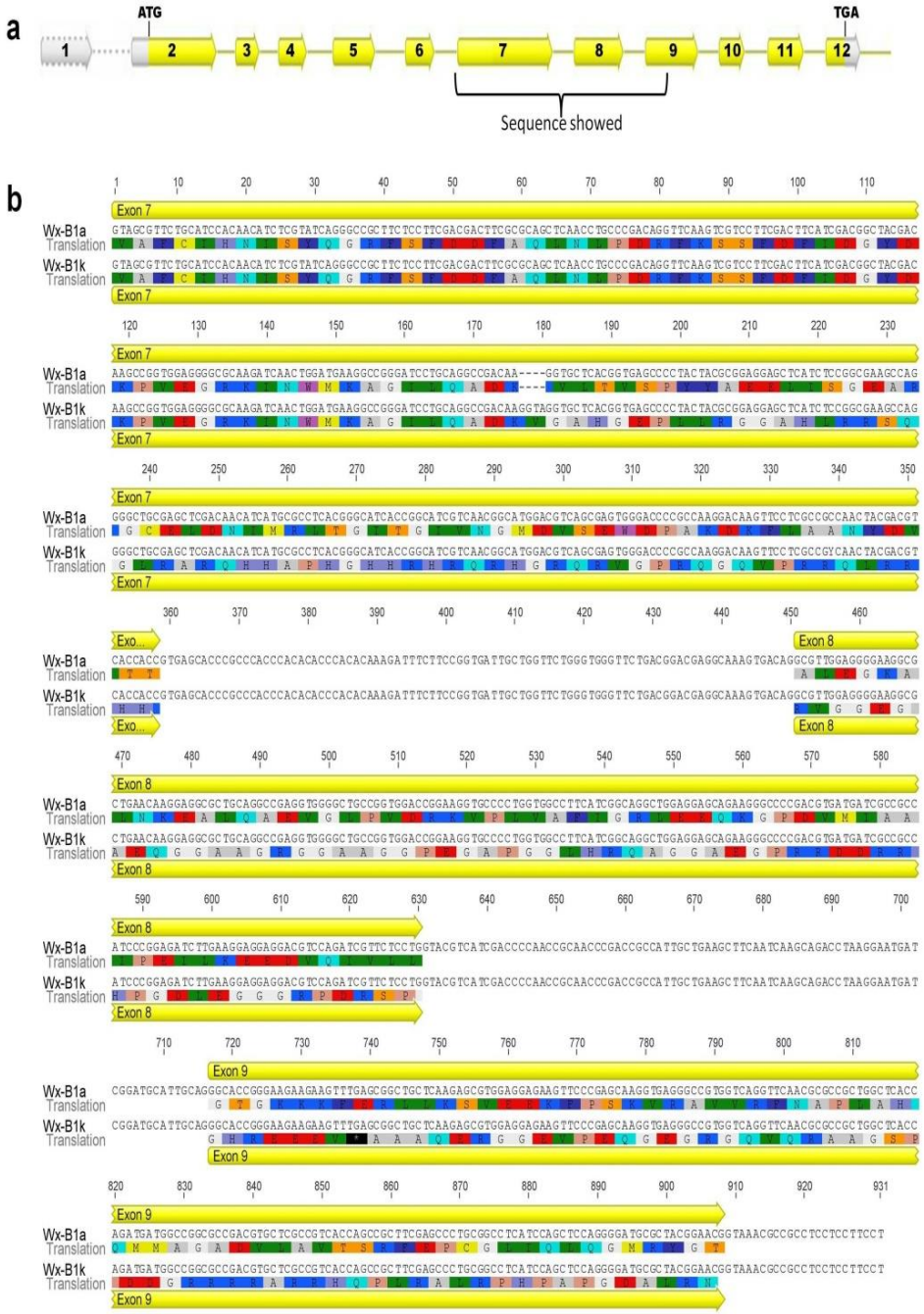


**Figure 3.** Molecular characterization of the novel *Wx-B1k* null allele. **a**, Diagrammatic representation of the *Wx-B1* gene. *Yellow* regions encode waxy protein. *Dotted* regions are not analysed. **b**, Comparison of genomic DNA sequences and deduced amino acid sequences of *Wx-B1a* and *Wx-B1k* alleles.

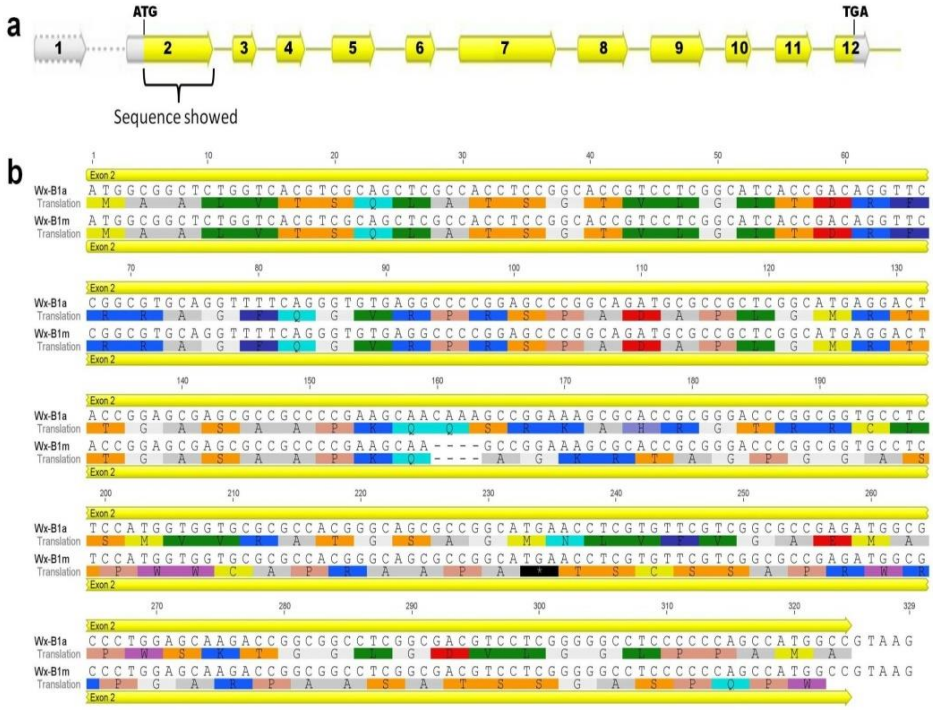


**Figure 4.** Molecular characterization of the novel *Wx-B1m* null allele. **a**, Diagrammatic representation of the *Wx-B1* gene. *Yellow* regions encode waxy protein. *Dotted* regions are not analysed. **b**, Comparison of genomic DNA sequences and deduced amino acid sequences of *Wx-B1a* and *Wx-B1m* alleles.

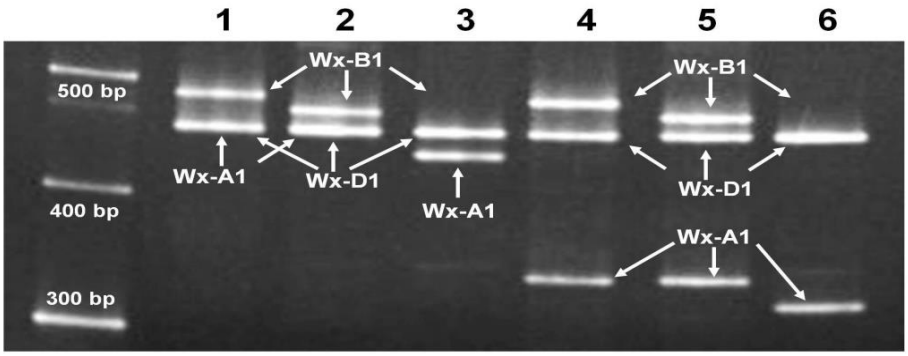


**Figure 5.** Molecular marker to detect *Wx-B1m* allele. In *lanes 1-3* PCR products resulted from the amplification with primers Wx1-Fw and Wx1.3Rv. In *lanes 3-6* the amplification products are digested with endonuclease *Dde*I, which has target sequences in *Wx-A1* amplicon. *Lanes* are as follow: *1* and *4* cv. Chinese Spring (*Wx-B1a*); *2* and *5* PI 442911 (*Wx-B1m*); and *3* and *6* cv. Kanto 107 (*Wx-B1b*).

The use of the primers designed by Nakamura et al. (2002) permits a rapid identification of the wheat lines that carry the *Wx-B1k* allele. The lines carrying this allele showed a *Wx-B1* band with reduced mobility (Fig. 2) compared to *Wx-B1a* due to the insertion of four nucleotides as described previously.

**Discussion**

The role of the *null Wx* alleles in the amylose content of wheat flour has been widely investigated in different studies; genotypes with the null allele for *Wx-B1* showed a greater decrease in amylose content compared to those with *Wx-A1* or *Wx-D1* nulls (Araki et al. 2000; Miura and Sugawara 1996; Yamamori and Quynh 2000). The detection of all null genotypes for the three *Wx* genes in modern wheat has been unsuccessful, with most detected genotypes null for *Wx-A1* or *Wx-B1* and in some rare occasions null for *Wx-D1* and both *Wx-A1* and *Wx-B1*. The available waxy wheat lines are products of crosses between partial-waxy genotypes in modern breeding. For bread wheat (Nakamura et al. 1995), the first successful cross was between two partial-waxy cultivars, Kanto 107 (*Wx-A1b*, *Wx-B1b* and *Wx-D1a*) and Bai Huo (*Wx-A1a*, *Wx-B1a* and *Wx-D1b*).

Other studies have since shown the existence of *null Wx* alleles derived from other genetic events (Guzmán et al. 2015; Saito and Nakamura 2005; Vanzetti et al. 2010). In the current study, two novel *null* alleles for *Wx-B1* were detected in a collection of two hexaploid wheat species, which enlarges the availability of *null Wx* alleles for wheat breeders. Paradoxically, some of these genetic materials were analysed by Li et al. (2013) showed differences to the variation detected in the current study.

Based exclusively on the use of PCR molecular markers, Li et al. (2013) reported the presence of numerous genotypes with *null Wx* alleles in macha wheat due to the absence of amplified PCR products. However our data,

based on the combined use of protein and DNA analysis, showed conflicting results with that of Li et al. (2013). These authors found in macha wheat two accessions (PI 361862 and PI 572911) with null alleles for the three waxy loci (*Wx-A1*, *-B1* and *-D1*), one accession (PI 572913) with *null* alleles for *Wx-B1* and *-D1*, and another one (PI 572910) with *null* alleles for *Wx-A1* and *-D1*. Additionally, two more macha accessions (PI 572906 and PI 290507) were described in their study as null for *Wx-D1* or for *Wx-B1*, respectively. It is important to mention that before the study of Li et al. (2013), no wheat accession with *null* alleles for all three *Wx* genes had been reported. This kind of wheat (waxy wheat, 0% amylose) has only been generated in breeding programs, as mentioned above (Nakamura et al. 1995; Yasui et al. 1997; Zhao et al. 1998). Besides, the *null* allele for *Wx-D1* is extremely rare. Yamamori et al. (1994) found one line lacking Wx-D1 protein in a collection of 1960 cultivars of different geographical origins (frequency of 0.05%). Guzmán et al. (2010) also identified one accession lacking Wx-D1 protein in a collection of 420 spelt lines (0.23%). However, Li et al. (2013) described five macha accessions having the *Wx-D1 null* allele in a total of 23 accessions analysed (21.73%). In the current study, all the macha wheat accessions described by Li et al. (2013) were analysed by SDS-PAGE. In contrast to their results, no polymorphism was found and all accessions showed three waxy proteins. The screening of the same collection with BDFL and BRD primers also confirmed the absence of *null* alleles (data not shown). The reason to explain why Li et al. (2013) obtained different results from ours is not known, but probably is due to false negatives that occurred in their PCR analysis, which led to misclassification.

A contrasting discrepancy was observed in the analysed accessions of Indian dwarf wheat. In this study, some accessions (PI 282451, PI 282452, PI 324492 and PI 352498) were found to carry the *Wx-B1b* null allele according to protein electrophoresis as well as PCR using the BDFL and BRD primers.

These results were in agreement with those of Li et al. (2013); however, they described PI 272580, PI 330556 and PI 352499 as having the wild allele for *Wx-B1*. We demonstrated here that these accessions had a novel *Wx-B1k* null allele by molecular characterization of the *Wx-B1* gene. The molecular characterization was carried out because the protein electrophoresis analysis (SDS-PAGE and 2D) revealed that these accessions lacked the Wx-B1 protein and this was not in agreement with the result obtained with the BDFL and BRD primers. This fact strengthens the idea that for appropriate evaluation of waxy protein variability, both protein and DNA analysis (PCR and/or sequencing) should be combined for waxy protein/gene alleles to avoid misclassification due to failures or inconclusive results with only method (Ortega et al. 2015).

In our study, the analysis of a set of club wheat showed five accessions lacking Wx-B1 protein, four of which had the *Wx-B1b null* allele as detected with BDFL and BRD primers. The other accession had a novel allele (*Wx-B1m*), characterized by the deletion of four nucleotides in the second exon. As for *Wx-B1k*, this produced a frameshift in the ORF (i.e. premature appearance of a stop codon) which would result in absence of the protein. This kind of frameshift mutation leading to lack of the protein has been described several times in *Wx-A1* (Saito et al. 2004; Saito and Nakamura 2005; Vanzetti et al. 2010), but only once in *Wx-B1* (Guzmán et al. 2015). This is important because the common *null* mutation for *Wx-B1* (*Wx-B1b*) implies the deletion of the entire gene and the surrounding region (67 kb), in which other genes related to quality could be included (Saito et al. 2009). Additionally, Wx-B1 protein has been shown to have a greater impact on amylose synthesis compared to Wx-A1 and Wx-D1 (Miura and Sugawara 1996; Yamamori and Quynh 2000), so the detection of variability is important to provide more sources of variation for starch modification.

Currently, several *null* alleles have been described that differ from the first ones (*b* alleles) described by Vrinten et al. (1999). Most molecular markers developed to screen for *Wx-1* gene variability (Li et al. 2013; McLauchlan et al. 2001; Nakamura et al. 2002) were designed to detect those first described *b null* alleles. The sole use of these markers could generate misclassification. In the case of the novel *Wx-B1k* allele, the BDFL and BRD primers could be used to detect it in breeding programs, due to the slightly reduced mobility of its amplicon compared with the wild allele *Wx-B1a*. We have designed a molecular marker for the rapid detection of the novel *Wx-B1m* allele in breeding programs.

In conclusion, this study demonstrated the importance of combining both protein and molecular characterization analysis for appropriate analysis of variability of waxy proteins. Several accessions lacking Wx-B1 protein were detected, with some caused by the common *Wx-B1b* null allele. Two novel *Wx-B1 null* alleles were identified in Indian dwarf and club wheat, which could be used to enlarge the genetic variability for this gene. The differential effects of these novel *null* alleles in wheat quality compared to *Wx-B1b* need to be studied in further research.

**Acknowledgements**

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## Supplementary

**ESM-1.** Waxy protein composition of the material evaluated according with the SDS-PAGE separation.

*Triticum aestivum* subsp. *macha*

| Accession | Cultivar / Line | Origin | Wx-A1 | Wx-B1 | Wx-D1 |
|---|---|---|---|---|---|
| PI 272554 | I-1-2710 | Hungary | a | a | a |
| PI 272555 | I-1-3544 | Hungary | a | a | a |
| PI 278660 | 2708 | United Kingdom | a | a | a |
| PI 290507 | I-1-2710 | Hungary | a | a | a |
| PI 352466 | Letshchumicum | Former Soviet Union | a | a | a |
| PI 355508 | 69Z5.187 | Former Soviet Union | a | a | a |
| PI 355509 | 69Z5.188 | Former Soviet Union | a | a | a |
| PI 355510 | 69Z5.189 | Former Soviet Union | a | a | a |
| PI 355512 | 69Z5.191 | Former Soviet Union | a | a | a |
| PI 355513 | 69Z5.192 | Former Soviet Union | a | a | a |
| PI 355514 | 69Z5.193 | Switzerland | a | a | a |
| PI 361862 | DN-2378 | Denmark | a | a | a |
| PI 428146 | G532 | Sweden | a | a | a |
| PI 428148 | G1260 | Russian Federation | a | a | a |
| PI 428177 | G524 | Sweden | a | a | a |
| PI 428178 | G866 | Italy | a | a | a |
| PI 542466 | H86-708 | United States | a | a | a |
| PI 572905 | WIR 29576 | Georgia | a | a | a |
| PI 572906 | WIR 28168 | Georgia | a | a | a |
| PI 572907 | WIR 28214 | Georgia | a | a | a |
| PI 572908 | H Tri 13595/89 | Georgia | a | a | a |
| PI 572909 | H Tri 13602/83 | Georgia | a | a | a |
| PI 572910 | H Tri 13603/89 | Georgia | a | a | a |
| PI 572911 | H Tri 13613/87 | Georgia | a | a | a |
| PI 572912 | H Tri 13615/87 | Georgia | a | a | a |
| PI 572913 | H Tri 13614/89 | Georgia | a | a | a |
| PI 611470 | H Tri 13601/87 | Georgia | a | a | a |

*Triticum aestivum* subsp. *compactum*

| Accession | Cultivar / Line | Origin | Wx-A1 | Wx-B1 | Wx-D1 |
|---|---|---|---|---|---|
| PI 25970 | Bola blanca | Mexico | a | a | a |
| PI 129523 | Ostka Skomoroska | Poland | a | a | a |
| PI 129528 | Sandomierka | Poland | a | a | a |
| PI 157920 | Velino | Italy | a | a | a |
| PI 159101 | Spitskop | South Africa | a | a | a |
| PI 191542 | H 23 H 13385 | Portugal | a | a | a |
| PI 211701 | 1413-1 | Turkey | a | a | a |
| PI 269250 | Terenzio 17077 | Italy | a | a | a |
| PI 278541 | Aleppo 23 | Syria | a | a | a |
| PI 278639 | American Club | United Kingdom | a | a | a |
| PI 278641 | ECH 1096 | United Kingdom | a | a | a |
| PI 294567 | Bivona | United States | a | a | a |
| PI 294891 | Anatolien | Bulgaria | a | a | a |
| PI 294892 | Herrison Sans Barbe | Bulgaria | a | a | a |
| PI 316807 | DN-2263 | Denmark | a | a | a |
| PI 330540 | Erinaceum | United Kingdom | a | a | a |
| PI 341432 | B-237 | Turkey | a | a | a |
| PI 352298 | Rouge de la Gruyere | Switzerland | a | a | a |
| PI 352299 | Rouge de la Gruyere | Switzerland | a | a | a |
| PI 352302 | Tiroler Fruhe Binkel | Austria | a | a | a |
| PI 352307 | Konya | Turkey | a | a | a |
| PI 352319 | Compactaoide 5 | Switzerland | a | a | a |
| PI 357307 | W 2691 | Australia | a | null | a |
| PI 366118 | D-11 | Egypt | a | a | a |
| PI 372151 | Vardenik 9 | Armenia | a | a | a |
| PI 377686 | Gluclub | Australia | a | a | a |
| PI 410652 | W44A | Pakistan | a | a | a |
| PI 410654 | W78B | Pakistan | a | a | a |
| PI 412975 | Knoppies Caledon | South Africa | a | a | a |
| PI 412979 | Malgas | South Africa | a | a | a |
| PI 422411 | Tincurrin | Australia | a | a | a |
| PI 434642 | Tincurrin | Australia | a | a | a |
| PI 442911 | Line AA | Australia | a | null | a |
| PI 442912 | Line F | Australia | a | null | a |
| PI 442913 | Line S | Australia | a | null | a |
| PI 554120 | M72-1250 | United States | a | a | a |
| PI 554121 | M85-9 | United States | a | a | a |
| PI 565431 | Club | United States | a | null | a |
| PI 566594 | Calorwa | United States | a | a | a |
| PI 572855 | 86PK1288-001.05 | Pakistan | a | a | a |
| PI 572856 | 86PK1291-001.05 | Pakistan | a | a | a |
| PI 597666 | WA 7770 | United States | a | a | a |
| PI 628641 | Chukar | United States | a | a | a |

*Triticum aestivum* subsp. *sphaerococcum*

| Accession | Cultivar / Line | Origin | Wx-A1 | Wx-B1 | Wx-D1 |
|---|---|---|---|---|---|
| CItr 4528 | Erinaceum | Pakistan | a | a | a |
| CItr 4529 | Linaza | Pakistan | a | null | a |
| CItr 4531 | Humboldti | Pakistan | a | a | a |
| CItr 4923 | 125 | India | a | null | a |
| CItr 8610 | 52 | China | a | a | a |
| CItr 17737 | - | United States | a | a | a |
| PI 115818 | 23824 | India | a | null | a |
| PI 182118 | S-49 | Pakistan | a | null | a |
| PI 191301 | Sahari M.3 | Portugal | a | a | a |
| PI 272580 | I-1-3572 | Hungary | a | null | a |
| PI 272581 | I-1-3573 | Hungary | a | null | a |
| PI 277141 | S 2130 | Germany | a | a | a |
| PI 277142 | Acarp | India | a | a | a |
| PI 277164 | Randhawai | Pakistan | a | null | a |
| PI 277165 | Rubroglabrum | Pakistan | a | a | a |
| PI 278650 | 971 | United Kingdom | a | a | a |
| PI 282451 | Cawnpore | India | a | null | a |
| PI 282452 | TRI 3008 | India | a | null | a |
| PI 324492 | - | India | a | null | a |
| PI 330556 | Echinatum | United Kingdom | a | null | a |
| PI 337997 | - | India | a | a | a |
| PI 352498 | Acarp | India | a | null | a |
| PI 352499 | Cawnpore | India | a | null | a |

# DISCUSIÓN GENERAL Y CONCLUSIONES

La existencia de variabilidad es la piedra angular de cualquier programa de mejora genética, dado que permite el desarrollo de nuevos materiales adaptados tanto a las cambiantes condiciones climáticas de las zonas donde esos materiales serán cultivados como a las demandas, igualmente variables, por parte de los consumidores. Esto último tiene gran importancia en aquellas zonas donde los procesos de mejora han permitido alcanzar óptimos rendimientos y donde se comienza a valorar, cada vez más, la diversificación de usos y el desarrollo innovador de productos alimenticios de mayores calidades nutricionales y más saludables.

En el caso del trigo, las tendencias actuales incrementan la necesidad de una búsqueda de fuentes de variación que permitan el desarrollo de materiales adaptados a una multitud de aplicaciones de la harina o sémola de trigo. A este respecto, los componentes del endospermo del grano de trigo implicados en las propiedades asociadas a la calidad harino-panadera o semolera, como proteínas de reserva, almidón o puroindolinas, son pieza fundamental en el desarrollo de nuevos cultivares.

Una forma de aumentar y diversificar estas propiedades es incrementando la base genética del cultivo mediante la incorporación de los genes implicados procedentes de todas aquellas fuentes de variación disponibles. Dentro estas fuentes, podemos encontrar todos aquellos materiales que, si bien hoy en día se encuentra catalogados como abandonados o infrautilizados, fueron utilizados en el pasado y que se encuentran almacenados y conservados en los Bancos de Germoplasma.

En esta Tesis Doctoral se han evaluado, con la finalidad de poner en valor la variación que contienen, una serie de colecciones de trigos hexaploides de diversa procedencia geográfica. Así, se han utilizado variedades criollas mexicanas, junto con variedades tradicionales u obsoletas

de Andalucía, de trigo harinero (*T. aestivum* ssp. *aestivum*) obtenidas las primeras del Banco de Germoplasma de CIMMYT, y las segundas tanto del Centro de Recursos Fitogenéticos del INIA (Alcalá de Henares, España) como de la National Small Grain Collection (NSGC) del Departamento de Agricultura de los Estados Unidos de América (USDA, Aberdeen, Idaho). A estos materiales se han añadido las colecciones disponibles en la NSGC de trigo cabezorro o club (*T. aestivum* ssp. *compactum*), enano de la India (*T. aestivum* ssp. *sphaerococcum*) y trigo macha (*T. aestivum* ssp. *macha*).

La caracterización de alguna de estas colecciones ya había sido iniciada en trabajos previos del grupo de investigación. En el caso de las variedades criollas mexicanas su morfología y variación para proteínas de reserva fue analizada por Caballero et al. (2010) y la variación de las proteínas del almidón por Guzmán et al. (2015). Por ello, en estas Tesis se abordó la cuestión de la textura y la variación para puroindolinas. Los resultados mostraron que los alelos presenten en estos materiales para ambas puroindolinas ya habían sido previamente descritos. Sin embargo, es de destacar que algunos de ellos, en concreto *Pinb-D1e*, son considerados raros debido a su escasa frecuencia en los materiales evaluados por otros autores (Morris et al. 2001). Además, debido a que estos materiales fueron introducidos por los colonizadores en México a partir de trigos cultivados en España, se observó que la distribución de estas variantes raras es consecuencia de la acción de procesos de deriva genética, lo cual es común en materiales donde la intervención de los agricultores está asociada a su dispersión.

En el caso de las variedades tradicionales procedentes de diferentes provincias de Andalucía, este tipo de materiales aparecen en las zonas menos adaptadas agronómicamente para el cultivo actual de trigo en esta región española, y han sido asociados a la elaboración de productos tradicionales tipificados como es el caso de la IGP «Pan de Alfacar», en la cual se usaron

tradicionalmente los trigos cultivados en la zona de Guadix (Granada). La importancia de estos materiales no sólo radica en su uso *per se*, sino en la evaluación de sus características de calidad tecnológica que puedan servir como indicadores para la selección de materiales modernos con estas características, pero mejor adaptados agronómicamente. A este respecto se evaluó tanto su morfología, según los parámetros botánicos usados en la clasificación del herbario «*Ceres Hispanica*» de Lagasca y Rojas Clemente (Téllez Molina y Peña 1952), sino también su composición para proteínas de reserva (HMWGs y LMWGs) y puroindolinas. Para estas últimas, la variación detectada fue baja, sin presentar variantes no detectadas en otro tipo de materiales, si bien algunas de estas variantes si pueden considerarse como poco frecuentes, lo cual hace que estas variedades tradicionales sean interesantes como fuentes alternativas de esta variación. En el caso de las HMWGs, la utilización de geles de poliacrilamida con y sin urea en electroforesis vertical si ha permitido detectar nuevas variantes alélicas. No obstante, a pesar de la variabilidad encontrada y las nuevas variantes encontradas, algunos de estos alelos detectados presentaron muy baja frecuencia, mientras que otros fueron claramente hegemónicos.

Es bien conocido que en el pasado, el cultivo del trigo incluía un número considerablemente mayor de especies y subespecies del género *Triticum*, tanto a nivel tetraploide como hexaploide. La búsqueda de diversificación ha generado desde hace años la recuperación de algunos de estos materiales y su impulso como cultivos alternativos a veces ligados a consideraciones nutricionales no siempre bien contrastadas. En estos materiales, podemos encontrar al Kamut© (una variedad registrada - QK-77- de *T. turgidum* ssp. *turanicum* Jakubz. em A. Löve & D. Löve), al farro (o emmer cultivado) o al espelta. Todo ello abre la posibilidad de evaluar otras subespecies abandonadas o infrautilizadas como es el caso de las subespecies de *T. aestivum* que se han utilizado en la presente Tesis Doctoral. En este

caso, su evaluación se ha constreñido a la variación de las proteínas waxy, dentro de las cuales se han detectado, además de varios alelos descritos previamente por otros autores, dos variantes nulas (no presencia de la proteína waxy) del gen *Wx-B1* que muestran la presencia de la secuencia nucleotídica, y que por tanto no corresponden con las variantes nulas descritas hasta ahora asociadas a la deleción del gen.

Todo ello ha permitido obtener las siguientes conclusiones:

1. Las variedades tradicionales puede ser óptimas fuentes de variación para puroindolinas no sólo por la presencia de nuevos alelos, sino por incluir variantes alélicas poco frecuentes.
2. La variación mostrada en morfología y composición del grano de las variedades locales de Andalucía evidencia una gran diversidad, principalmente localizada en los materiales de Granada y Almería, que podría estar asociada a los usos tradicionales de estos materiales en dichas zonas geográficas.
3. El análisis de la variabilidad para proteínas waxy debe realizarse combinando la separación electroforética de las proteínas en sistema uni- y bi-dimensional junto con la amplificación alelo específica mediante PCR.
4. Estos resultados evidencian la necesidad de protección y conservación de estas colecciones, dado que la variabilidad detectada podría no encontrarse en otros materiales.
5. En el contexto actual de resurgimiento de los productos tradicionales, la variabilidad de los materiales evaluados podrían ser utilizada tanto para su recuperación como cultivo *per se* como para su uso en programas de mejoramiento, además de ofrecer rasgos de calidad deseados para la elaboración de los alimentos tradicionales.

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