Original article

Waxy genes from spelt wheat: new alleles for modern wheat breeding and new phylogenetic inferences about the origin of this specie.

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Running tittle: Spelt *waxy* genes: new alleles and phylogenetic inferences.

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1 **Abstract**

2 *Background and aims:* Waxy proteins are the enzymes responsible for amylose
3 synthesis in wheat seeds, being encoded by three *waxy* genes (*Wx-A1*, *Wx-B1* and *Wx-*
4 *D1*) in hexaploid wheat. In addition to the interest shown in these genes due of their
5 effect on starch quality, *waxy* loci have been used to study the phylogeny of wheat. The
6 origin of European spelt (*Triticum aestivum* ssp. *spelta)* is not clear. This study used
7 molecular characterization to investigate the *waxy* genes in a Spanish spelt collection
8 and compare them with their homologous genes in emmer (*T. turgidum* ssp. *dicoccum),*
9 durum (*T. turgidum* ssp. *durum)* and common wheat (*Triticum aestivum* ssp. *aestivum*),
10 together with other Asian and European spelt that could be used to determine the origin
11 of European spelt.

12 *Materials and methods: waxy* genes were amplified and sequenced. Sequences were
13 analysed by Geneious Pro software; nucleotide diversity was analysed by DNAsp and
14 MEGA5 was used for the phylogenetic analysis.

15 *Key results:* Three, four and three new alleles were described for the *Wx-A1*, *Wx-B1* and
16 *Wx-D1* loci, respectively. The results of this study led to the classification of spelt into
17 two groups based on the variation in the *Wx-B1*, which suggests that there were two
18 different origins for the emmer wheat that has been found to be part of the spelt genetic
19 makeup. One of these groups was only detected in Iberian material. No differences were
20 found between the rest of the European spelt and the Asiatic spelt, which suggested that
21 the Iberian material had a different origin from the other spelt sources.

22 *Conclusions:* Results suggested that the *Wx* gene variability present in wheat could be
23 undervalued because some of these variants were not detected by traditional
24 classification based on SDS-PAGE. The evaluation of this variability has permitted the

detection ten new *waxy* alleles that could affect starch quality and thus they could be used in modern wheat breeding. Besides two different classes of *Wx-B1* were detected that could be used for evaluating the phylogenetic relationships and the origins of different types of wheat.

Keywords: molecular characterization, phylogeny, spelt origin, *waxy* genes.

Introduction

The granule-bound starch synthase (GBSSI), or waxy protein, is the enzyme responsible for the synthesis of amylose in wheat grain. As some important technological starch properties, such as gelatinization, pasting and gelation, depend on the amylose:amylopectin ratio (Zeng et al. 1997), waxy protein has been the subject of many studies in recent years. Three waxy proteins are present in hexaploid wheats, which are encoded by three genes: *Wx-A1* (located on chromosome 7AS), *Wx-B1* (chromosome 4AL translocated from the original 7BS) and *Wx-D1* (chromosome 7DS) (Yamamori et al. 1994);each consists of 11 exons and 10 introns as Murai et al. (1999) showed sequencing the three genes, coding for three peptides of 604, 605 and 604 amino acid residues respectively. Given the minor differences noted in their exon sequences, the molecular weights of the three proteins are very similar, this has meant that it has been very difficult to identify allelic variants among them. However, polymorphism studies have been carried out on durum wheat (Yamamori et al. 1995) and on common wheat (Rodríguez-Quijano et al. 1998), permitting the detection of different alleles for these genes, including null ones, which have been used as a base for breeding programmes focused on the production of amylose-free wheat (Nakamura et al. 1995; Kiribuchi-Otobe et al. 1997).

More recently the search for new alleles has been extended to ancient wheats (Urbano et al. 2002; Caballero et al. 2008a; Guzman et al. 2009, 2010, 2011), which have become very important in the search for genes that could be useful in modern wheat breeding programmes since modern agricultural practices have reduced the genetic variability of cultivated wheats. One of these is spelt (*Triticum aestivum* ssp.

spelta L. em. Thell.; 2n = 6× = 42, AABBDD), a currently minor crop that was widely cultivated in the past (Nesbitt and Samuel 1996). Recently it has undergone a revival associated with the health food market and low-input agriculture as this crop can grow without pesticides in marginal areas (Cubadda and Marconi 2002). Due to the renewed interest, a large collection of Spanish spelt has been analysed for traits related to the use-quality of this species. Caballero et al. (2001, 2004a, b) found considerable variability between seed storage proteins in the Spanish spelt collection. Some of these allelic variants showed an association with high gluten strength (Caballero et al. 2008b). Another important aspect, the starch quality, has been also analysed in this collection through polymorphism assessment of waxy proteins by SDS-PAGE (Guzman et al. 2010). Although some variation was found, most of these accessions (69.52%) were found to have the *Wx-A1a, Wx-B1a* and *Wx-D1a* alleles, which have been catalogued as wild in common and durum wheats. Nevertheless, due to the small differences in size between the waxy proteins, it is possible that the true variability could be higher, mainly through internal differences in the amino acid sequences. This increased variability could generate enzymes with different degrees of functionality. Further evaluation of the accessions that show these wild alleles using molecular characterization could lead to the identification of these mimetic alleles.

Another interesting question about this crop that has caused a certain amount of controversy is the origin of the spelt grown in Europe. Two main hypotheses have been suggested. The first hypothesis is that European spelt is an ancestor of common wheat that spread from Asia; while the other suggests that Asian spelt is the ancestor of common wheat but European spelt had an independent origin and is derived from a secondary hybridization between emmer wheat (*T. turgidum* ssp. *dicoccum* Schrank)

1 and a cultivated hexaploid wheat, probably *T. aestivum* L. ssp. *compactum* Host em.
2 Mackey (Liu and Tsunewaki 1991; Yan et al. 2003; Blatter et al. 2004; Dedkova et al.
3 2004). More recently, Dvorak et al. (2012) showed that also some forms of the Asiatic
4 spelt could have their origin in free-threshing wheat. In addition to this, other authors
5 (Dedkova et al. 2004, Elia et al. 2004) have suggested that European spelt could be
6 classified into two eco-geographical groups: the Iberian (pol. *ibericum* Flaskb.) and the
7 Bavarian (pol. *bavaricum* Vav.) geographical groups. These authors suggested that
8 Iberian spelt could have its origin in Asia and be different to the other types of European
9 spelt. *Waxy* gene sequences have been utilized to study the origin and phylogeny of
10 other *Poaceae* species, including wheat (Mason-Gamer et al. 1998, Yan et al. 2000,
11 2003; Mason-Gamer 2001; Yan and Bhave 2001; Ingram and Doyle 2003; Fortune et al.
12 2007), and consequently, the characterization of the *waxy* genes in this Spanish spelt
13 collection could shed light on this question about the origin of Spanish spelt.
14 The aim of the current study was the molecular characterization of the *waxy* genes
15 present in this Spanish spelt collection, and compares them with their homologous
16 genes in emmer, durum and common wheat, together with other spelt examples of
17 Asian and European origin that could be used for phylogenetic studies on the origin of
18 European spelt.
19
20 **Material and Methods**
21 *Plant material*
22 Six Spanish and two Iranian spelt accessions obtained from three Germplasm
23 Banks were analysed first for waxy proteins composition. The six Spanish accessions
24 (PI 348458, PI 348471, PI 348489, PI 348515, PI 348595, PI 3487447) were from the

National Small Grain Collection (Aberdeen, USA) while the Iranian accessions (CGN 8384 and CGN12269) were obtained from the Center for Genetic Resources (Netherlands). One more spelt accession (CGN11460, Czech Republic origin) and one emmer German accession (CGN16104) were used also in sequencing analysis.

SDS-PAGE analysis

The waxy proteins were extracted from starch granules of mature seeds following the method of Echt and Schwartz (1981) and separated by SDS-PAGE using 12 % polyacrylamide gels with low bys-acrylamide concentration (C: 0.44%).

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. DNA was isolated using the DNAzol® method (Invitrogen, Carlsband, CA, USA).

The primers designed by Monari et al. (2005) were used to amplify the central region (region spanning from the middle of exon 3 to exon 6) of the *waxy* genes: WxBAF (5’-ACTTCCACTGCTACAGCGCGGGGT-3’), WxBAR (5’-GCTGACGTCCATGCCGTTGACGATG-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 0.75U DNA polymerase (Promega, Madison, WI, USA). 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, 2 min at 62°C then 1 min 5 s at 72°C. After the 35 cycles a final extension of 5 min at 72°C was done. Additionally, the PCR products were restricted with the endonuclease *Bgl*I (TaKaRa) following the supplier’s instructions.

1 Amplification and digested products were fractionated in vertical PAGE gels with
2 8% polyacrylamide concentration (w/v, C: 1.28%), and the bands were visualized by
3 ethidium bromide staining.
4
5 *Cloning of waxy genes and sequencing analysis*
6 To clone almost the entire sequence of the waxy genes, besides primers WxBAF
7 and WxBAR described above, pair of primers WxF3 (5'-
8 TCTGGTCACGTCCCAGCTCGCCACCT-3')/WxVT1R (5'-
9 ACCCCGCGCTTGTAGCAGTGGAAGT-3') (Tm = 64°C) and WxVT1F (5'-
10 CATCGTCAACGGCATGGACGTTCAGC-3')/ WxVTR (5'-
11 CCAGAAGCACGTCCTCCCAGTTCTTG-3') (Tm = 64°C) were used to amplify the
12 beginning and the end of the *waxy* genes respectively. PCR products were excised from
13 polyacrylamide gel and cloned into pGEM T-easy vector (Promega, Madison, WI,
14 USA) for sequencing. Inserts were sequenced from at least three different clones using
15 an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Carlsban, CA, USA).
16 The sequences reported in the current study were compared to the sequences
17 available in the GenBank database using Geneious Pro ver. 5.0.3 software (Biomatters
18 Ltd.).
19
20 *Data analysis*
21 DNA analyses were conducted by DNAsp ver. 5.0 (Librado and Rozas 2009).
22 Nucleotide diversity was estimated as theta (θ), the number of segregating
23 (polymorphic) sites (Watterson 1975), and pi (π), the average number of nucleotide

differences per site between two sequences (Nei, 1987). Tests of neutrality were performed using Tajima's *D* statistic (1989).

The synonymous (*Ks*) and non-synonymous (*Ka*) substitution rates and the relation *Ka/Ks* were computed using DNAsp ver. 5.0 (Librado and Rozas 2009). Divergence times were calculated by the mean divergence times (2.7 million years ago, MYA) between the A and D genome estimated by Dvorak and Akhunov (2005).

Phylogenetic tree was constructed with software MEGA5 software (Tamura et al. 2011) using the complete coding regions. Neighbour-joining cluster with all sequences analysed was generated using the Maximun Composite likelihood method (Tamura et al. 2004) and one bootstrap consensus from 1000 replicates was used (Felsenstein 1985).

Results

Electrophoretic analysis

As shown by previous results obtained with this collection (Guzman et al., 2010), no differences could be detected by SDS-PAGE between the different Spanish spelt genotypes and the common wheat (cv. Chinese Spring) used as a standard (Fig. 1). The data obtained by uni-dimensional SDS-PAGE separation of the waxy proteins showed that the Spanish spelt (Fig.1a, lanes 1-2, 5-8) had the same pattern as the common wheat (cv. Chinese Spring), which presented the wild alleles for all three genes (*Wx-A1a, Wx-B1a* and *Wx-D1a*). Furthermore, the analysis included two Asiatic spelts of Iranian origin that showed the same waxy proteins pattern (Fig. 1a, lanes 3-4). This similarity was also observed when the samples were separated by 2D-IEF × SDS-PAGE separation (Fig. 1b and 1c).

However, the comparison of these supposed wild alleles by PCR amplification of

1 genomic DNA and posterior electrophoretic migration, showed two groups within the
2 genotypes analysed. The most important differences between the samples analysed were
3 detected when the central region of the waxy genes was amplified (Fig. 2a). In this
4 figure, the samples used were the same as the ones shown in Figure 1, and appear in the
5 same order. Two Spanish and two Iranian spelt samples showed three bands (Fig. 2a,
6 lanes 1-4), each one corresponding to one of the three identified *waxy* genes, of the
7 expected size of: *Wx-D1* (1,017 bp), *Wx-A1* (958 bp) and *Wx-B1* (935 bp), based on the
8 published sequences of *Wx-A1a, Wx-B1a* and *Wx-D1a* alleles of cv. Chinese Spring
9 (Murai et al. 1999).This fact meant that they were similar to the common wheat (cv.
10 Chinese Spring; Fig. 2a, lanes 1-3, 4 and 5, respectively). However, the other spelt
11 samples (Fig. 2a, lanes 5-8) had only two bands, as the *Wx-A1* and *Wx-B1* bands had
12 comigrated. The first group was called spelt type I and the second, spelt type II.
13 To clarify these band patterns and to confirm the presence of the two spelt groups,
14 the PCR products were digested using the endonuclease, *Bgl*I. Based on the published
15 sequences for common wheat (cv. Chinese Spring), only one sequence in the *Wx-B1*
16 gene was targeted. Two bands of 897 bp and 38 bp were expected from the digestion of
17 *Wx-B1* spelt I gene. While the 897 bp band was found in all samples, the 38 bp band
18 was lost due to its small size (Fig. 2b). In the spelt II lines, one additional band had
19 separated from *Wx-A1* band after the digestion (Fig. 2b, lanes 5-8), confirming the
20 presence of a larger *Wx-B1* band that had comigrated with the *Wx-A1* band shown in
21 Fig. 2a.
22
23 *Sequences of spelt waxy genes and deduced proteins*
24 In order to characterize the variation found by electrophoretic methods, the three

1 *waxy* genes from each Spanish spelt type (I and II) and the two Iranian samples were
2 sequenced. *Waxy* genes from one European spelt accession and one emmer accession
3 were also sequenced. All these sequences were uploaded to the GenBank database
4 (Table 1). Comparisons between these sequences and others from emmer, durum and
5 common wheat previously available in GenBank were undertaken. The criteria used to
6 choose these materials was to compare potential material with the different origins of
7 the spelt (Asian and European) and the putative progenitors that may be involved in the
8 different cross events that generated spelt (emmer and common wheat).
9 A summary of DNA polymorphism found in the *Wx* sequences evaluated is shown
10 in Table 2. These data were evaluated using both the complete sequence (exons +
11 introns) and just the coding sequence (exons). In both cases, the *Wx-B1* gene displayed
12 the highest level of SNPs among the three genes with 163 polymorphic sites in the
13 complete sequence and 53 in the coding region (Table 2). In the latter, 36 of these
14 nucleotide substitutions were synonymous (silent mutations) while the rest were non-
15 synonymous, which implied changes in the amino acid sequences. The ten alleles
16 identified encoded seven different polypeptides (Table 3). This polymorphism was clear
17 lower down in the coding regions of the other two genes (Tables 2 and 3). The *Wx-A1*
18 gene coding region showed 15 nucleotide substitutions (eight synonymous and seven
19 non-synonymous) while the *Wx-D1* gene only presented two substitutions that were
20 synonymous. Consequently, only four and one polypeptides were obtained for the seven
21 and two alleles detected in the coding regions of the *Wx-A1* and *Wx-D1* genes,
22 respectively (Table 3).
23 Two statistics, π (Nei 1987) and θ (Watterson 1975), were used to estimate
24 nucleotide diversity (Table 2). Both values were similar in all the cases that were

1 associated with a drift-mutation balance. The values for the *Wx-B1* gene were higher
2 than in the other two genes. Tajima's *D* values were not significant for the three genes,
3 which was consistent with a neutral equilibrium (Table 2). The value was negative for
4 the *Wx-A1* gene, indicative of an excess of low frequency alleles, while the other two
5 genes showed positive values, which indicated an excess of intermediate frequency
6 alleles.

7 The data shown in Table 3 suggested that the previous classification of waxy
8 genes in Spanish spelt was incorrect. In all cases, only the class 1 DNA and protein
9 sequences corresponded with the wild allele for each locus. For the *Wx-A1* gene, six
10 classes, previously classified as *Wx-A1a*, could be reclassified because the nucleotide
11 sequences were different from those in the *Wx-A1a* allele present in common wheat.
12 However, four of them synthesised a polypeptide with the same amino acid sequence,
13 which included all the Spanish spelt types evaluated (Table 3).

14 The *Wx-D1* gene showed up to four different nucleotide sequences, although only
15 two classes were observed when the coding region was analysed. In all cases, one
16 unique polypeptide was synthesised but the classification of this gene must indicate the
17 presence of the four alleles (Table 3).

18 The differences in the *Wx-B1* gene were larger than the other two genes. Up to
19 seven nucleotides sequences were detected in the materials previously classified as wild
20 (*Wx-B1a*). Six were different to the true *Wx-B1a* allele (class 1) and their translation
21 indicated the presence of four additional polypeptides. Two of these polypeptides were
22 associated with each one of the classes detected in the Spanish spelt (type I and II). It is
23 important to emphasise that although the nucleotide sequences were clearly different,
24 one of these two polypeptides was the same as those detected previously in the BGE-

012302 accession of the emmer that had presented the *Wx-B1g* allele (Guzman et al. 2011).

With respect to the Spanish spelt lines evaluated in the current study, the spelt type I had a homology of 99.5% for the *Wx-A1* gene compared to the *Wx-A1*gene from common wheat. Spelt type II also showed a homology of 99.6% with respect to spelt type I and common wheat, although the SNPs were not the same when compared to spelt type I or to common wheat. Specifically, the deduced Wx-A1 protein from the two spelt types was similar and showed three amino acid differences compared to the Wx-A1 protein from common wheat (*Wx-A1a* allele): Phe 60 → Gly, Asp 61 → Asn and Trp 454 → Arg. This meant that two alleles were identified within the allele previously catalogued as *Wx-A1a*. A third polypeptide was detected in the wild emmer that contained two amino acid changes (Glu 333 → Gly and Trp 454 → Arg), while a fourth polypeptide was observed in one German spelt and one Iranian emmer that showed two additional differences compared with the Wx-A1 protein from common wheat: Met 42 → Ile and Ser 75 → Cys. However, for the *Wx-D1* gene, both types of spelt showed a similarity of 99.9% to common wheat at the DNA level and no variation was detected in the protein sequences.

In the case of the *Wx-B1* gene, the differences detected were remarkable. Most of the differences observed were found in introns but 53 of the 163 SNPs were also present in the coding region, which generated up to ten *Wx-B1* alleles. The Spanish spelt lines divided into two different groups. Spelt type I presented one polypeptide corresponding to the *Wx-B1g* allele detected in the BGE-012302 accession of Spanish emmer, although the nucleotide sequences showed some differences between them. One similar case was observed with spelt type II and cv. Langdon. The 14 amino acid differences between

1 both types of polypeptides were notable (Fig. 3): Ser 34 → Asn, Pro 39 → Ala, Gly 41
2 → Val, Thr 45 → Ile, Gln 54 → -, Thr 62 → Ser, Ala 76 → Gly, Ser 246 → Asn, Arg
3 250 → Met, Ala 358 → Thr, Ala 365 → Val, Ser 451 → Asn, Met 510→ Val, Glu 554
4 → Gly. In a previous study based on protein analysis (Yamamori et al. 1995), the *Wx*
5 allele present in cv. Langdon was classified as *Wx-B1a*, probably due to a similar
6 problem as the one that arose with the Spanish spelt in the current study. However, the
7 current analysis showed that both alleles were very different. Spelt type I lines showed
8 only one difference with respect to common wheat: Arg 520 → His.
9 In summary, analyses of the Spanish spelt lines that were previously classified as
10 wild genotypes (*Wx-A1a*, *Wx-B1a*, *and Wx-D1a*) have shown that, although they
11 presented one unique allele for the *Wx-A1* gene, this was not the *Wx-A1a* allele.
12 Furthermore, the Spanish spelt lines showed two different alleles for the *Wx-B1* gene,
13 neither of which were the *Wx-B1a* allele.
14
15 *Phylogenetic analysis*
16 A phenogram based on the Maximum Composite Likelihood method was
17 constructed using all the *waxy* sequences evaluated in this study, together with the
18 putative donors from the genomes present in tetra- and hexaploid wheats (Fig. 4). *T.*
19 *urartu* Thum. ex Gandil (A^uA^u, *Wx-*A^u*1:* JN857937) and einkorn (*T. monococcum* L.
20 ssp. *monococum*; A^mA^m, *Wx-*A^m*1*: AF110373) were included from the A genome;
21 *Aegilops speltoides* Tausch. (S^sS^s, *Wx-*S^s*1*: AF110374) was included from the B genome
22 and *Ae. tauschii* Coss. (DD, *Wx-D1*: AF110375) was included from the D genome. The
23 total nucleotide sequence (exons + introns) was used in all cases.
24 Seven groups were observed in the dendrogram (Fig. 4). Two of them

1 corresponded with the *Wx-A1* and *Wx-D1* genes, respectively. With respect to the
2 putative donors from the wheat genomes, only the *Wx-D1* gene from *Ae. tauschii*
3 showed a narrow relationship with the D genome. The other three species were clearly
4 separated from the genomes with which they had been associated. Both species with the
5 A genome (*T. urartu* and einkorn) were clearly separated from the *Wx-A1* gene present
6 in the tetra and hexaploid wheats evaluated in the current study.

7 Another important result was that the *Wx-B1* gene showed two separated groups.
8 Both groups have been associated with different types of the *Wx-B1* gene detected in the
9 spelt groups (type I and II). Inside each group, the differences between nucleotide
10 sequences were small compared with the differences detected between groups.

11 The *Ks* and *Ka* substitution rates among *Wx* genes both for homeologous (*Wx-A1*,
12 *Wx-B1* and *Wx-D1*) as for orthologous (*Wx-B1* type I and *Wx-B1* type II) were
13 calculated by using the coding sequence of the complete gene. The comparison values
14 of the homeologous genes were ≈ 3-fold than the value obtained for the two types of
15 *Wx-B1* genes (Table 4). Consequently, the divergence time between both *Wx-B1* gene
16 types was estimated in ≈ 0.6 MYA (Table 4), based in the mean divergence time for the
17 separation between the A and D genome (2.7 MYA) estimated by Dvorak and Akhunov
18 (2005).

19

20 **Discussion**

21 The major nutritional component in wheat grains is starch, which is formed by
22 two glucose polymers: amylose and amylopectin, whose synthesis involves up to five
23 starch synthases (Baldwin 2001). The variation in the ratio between both polymers,
24 together with their chemical properties, is important for defining the end use of a

specific wheat flour type. Starch with high amylose content could be used to create healthier foods because the amylose is digested more slowly in the small intestine, providing beneficial effects for human health (Topping and Clifton 2001). However, wheat containing amylose-free starch has been reported to improve noodle quality (Oda et al. 1980) and be more efficient than standard wheat if the grain is used as a substrate for bioethanol production (Wu et al. 2006). Consequently, the search for different forms of starch synthases has increased so that new resources can be made available for breeding programmes focused on starch properties.

In the last 20 years, the waxy proteins have been the main subject of many studies, mainly focused on the search for null forms of these proteins or mutants showing less activity (Yamamori et al. 1994; Rodríguez-Quijano et al. 1998; Yanagisawa et al. 2001). However, the characterization of the apparently functional enzymes has been less studied because the dosage effect of these proteins makes it difficult to evaluate the specific effect of each variant on the amylose content. In this context, the current study characterized, at a molecular level, the *waxy* genes from different spelt accessions that had not shown any differences when they were previously evaluated for waxy protein polymorphism by SDS-PAGE (Guzman et al. 2010).

Although other authors have found high levels of nucleotide diversity for these genes in common wheat (Huang and Bûlé-Babel 2012), it is important to emphasise that the allelic variation found in the current study was detected in materials that were previously classified as similar when they were analysed by SDS-PAGE. This suggested that a greater part of the *Wx* gene variation could have emerged due to the similarity between the different synthesised proteins. However, the variation found in the nucleotide sequences was considerably higher. Seven, ten and four different alleles for

1 the *Wx-A1*, *Wx-B1* and *Wx-D1* genes, respectively, were detected where only one
2 polypeptide was detected by SDS-PAGE for each gene. This variation at the DNA level
3 was finally confirmed by the existence of 3 and 6 novel waxy alleles for the Wx-A1 and
4 Wx-B1 proteins respectively. The new alleles detected could have a different
5 functionality than the wild type ones and thus affect the composition
6 (amylose/amylopectin) and functionality of starch. This fact should be tested in further
7 experiments transferring these alleles to the same genetic background and evaluating
8 their use for modern wheat breeding.
9 In addition to the potential improvements that these mutations could have on
10 starch quality, variation in the nucleotide sequence of the *Wx* genes has been identified
11 as a useful tool in phylogenetic analysis (Mason-Gamer et al. 1998, Yan et al. 2000;
12 Mason-Gamer 2001; Ingram and Doyle 2003; Fortune et al. 2007). The *Wx-A1*, *Wx-B1*
13 and *Wx-D1* spelt sequences were compared to the homologous genes in the putative
14 donors of A, B and D genomes in polyploid wheats to shed more light on the origin of
15 each of the genomes. The current data only supported the hypothesis that suggested that
16 *Ae. tauschii* is the D genome donor (Dvorak et al. 1998), whereas there was no data to
17 support the second hypothesis about the origin of the A and B genomes. The current
18 theory on the origin of the A genome in hexaploid wheat suggests that the wild wheat
19 species (*T. urartu*) could be the A genome donor (Dvorak et al. 1993). However, the *Wx-*
20 *A^u1* sequence obtained in a previous study (Guzman and Alvarez 2012) was clearly
21 different to the rest of the *Wx-A1* sequences (Fig. 4), with the exception of the *Wx-A^m1*
22 gene sequenced in einkorn by Yan et al. (2000). Similar results were found when the
23 homology between the *Wx-S1* gene from *Ae. speltoides*, main candidate for the B
24 genome (Petersen et al. 2006), and the *Wx-B1* gene were evaluated. Although these

1 results should be treated with caution, due to the fact that only one sequence of *Ae.*
2 *speltoides* was used, this data suggested that the homology among these sequences was
3 low and this proposed origin was not clear based on the information derived from *waxy*
4 genes.

5 However, it is remarkable that the *Wx-B1* genes from the polyploid wheats
6 evaluated in this study clearly separated into two different groups. The *Wx-B1* type I
7 group had a greater similarity to the *Wx-S1* gene than the type II group. Although the
8 type II group had been associated with one durum wheat cultivar (c.v. Langdon), the
9 genealogy of this cultivar [Langdon = Carleton / Mindum // Khapli /3/ Carleton /
10 Mindum / Stewart /4/ Stewart] by Zeven and Zeven-Hissink (1976), indicated that it had
11 been generated using several lines of emmer wheat (Carleton = vernal emmer /
12 Mindum, Khapli = emmer and Stewart = Mindum /2* vernal emmer). This suggested
13 that there were two different origins for the emmer wheat that has been found to be part
14 of the spelt genetic makeup, although, to date, the second origin has only been found in
15 the Spanish spelt. These results confirm the unique nature of the Iberian spelt gene pool
16 indicated by other authors (Dedkova et al. 2004, Elia et al. 2004), who suggested that
17 Iberian spelt (pol. *ibericum* Flaskb.) could have its origin in Asia and be different to the
18 rest of the European spelts, which are said to derive from a secondary hybridization
19 between emmer and a cultivated hexaploid wheat, probably club wheat, as several
20 authors proposed (Liu and Tsunewaki 1991; Yan et al. 2003; Blatter et al. 2004;
21 Dedkova et al. 2004). This last hypothesis has not been totally verified (see Salamini et
22 al. 2002 for a review) as the current data has not revealed major differences between the
23 *Wx* sequences for the three genomes of European and Asian spelt evaluated. In the same
24 way, a recently hypothesis exposed by Dvorak et al. (2012) that suggest that all spelt

(except Iranian one) was certainly derived from a common hexaploid ancestor could not be proved with our data as the Iranian accessions analysed in this study were very similar to the rest ones. In addition to this we also found two different groups based on B genome differences, fact that could disagree with their hypothesis. The same authors also suggested that the hexaploid ancestor could have its origin in the cross of *Ae. tauschii* with a free-threshing tetraploid, and not emmer wheat. This fact could be also revised in further surveys with *waxy* data from more tetraploid species, as durum wheat. On the other hand, other authors have estimated the time of the origin of tetraploid wheat could be between 0.36 and 0.50 MYA (Dvorak and Akhunov 2005; Huang et al. 2002, respectively). Data obtained in the current study showed that the separation between both types of *Wx-B1* gene can have emerged during the synthesis of the tetraploid wheat. Although this hypothesis would be confirmed with further studies, this opens the possibility of that the origin of these wheats could be consequence of different events that would have effects on the origin of the spelt.

Further studies should be carried out in the future with these genes and others, with emmer, durum, spelt and common wheat accessions from as many locations as possible as well as the supposed ancestral genomes that may have contributed to tetraploid and hexaploid wheat (*T. urartu,* einkorn, *Aegilops* sp.) in order to clarify more the origin of spelt as well as the other cultivated wheats.

Conclusions

The first notable outcome from this study concerning the origin of Iberian spelt is that both spelt-I and spelt-II showed the same Wx-A1 protein sequence as emmer and durum wheat and varied only slightly with respect to common wheat. The results of this

study suggested a single origin for spelt and that common wheat developed subsequently. Nevertheless, the findings in relation to the *Wx-B1* genes supported the idea that, at least in the Iberian Peninsula, spelt could have a double phylogenetic origin.

The results from the current survey also suggested that the *Wx* gene variability seen in wheat could be undervalued because some of these variants were not detected by the traditional classification based in SDS-PAGE. The evaluation of this variability by the current study detected two different classes of *Wx-B1* gene, which could be used for evaluating the phylogenetic origins of and relationships between different wheat species.

Acknowledgements

This research was supported by grant AGL2010-19643-C02-01 from the Spanish Ministry of Economy and Competitiveness, co-financed with the European Regional Development Fund (FEDER) from the European Union. CG expresses his appreciation to Alessandra di Francesco, Andrea Aglieco, Tülin Sarigul and Ana Moral for their technical assistance.

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Figure legends

Figure 1. SDS-PAGE gel electrophoresis patterns of *waxy* proteins. a**,** uni-dimensional; and **b**, bi-dimensional separation. Lanes are as follow: **1,** PI-348471; **2,** PI-348489; **3,** CGN12269; **4,** CGN8384; **5,** PI-348515; **6,** PI-348458; **7,** PI-348747; and **8**, PI 348595. The cv. Chinese Spring (CS) was used as standard.

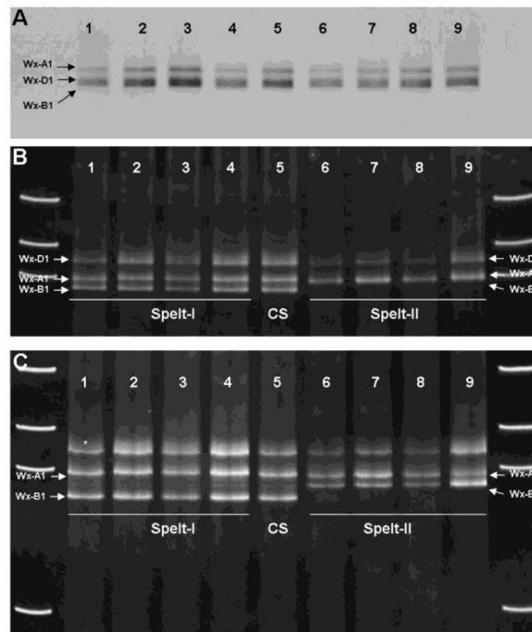

Figure 2. **a,** amplification products of the central region of the *waxy* genes; and **b**, amplification products of the central regions of the *waxy* genes digested with *BglI*. Arrow-heads indicates products of the digestion. Lanes are as follow: **1,** PI-348471; **2,** PI-348489; **3,** CGN12269; **4,** CGN8384; **5,** PI-348515; **6,** PI-348458; **7,** PI-348747; and **8**, PI 348595. The cv. Chinese Spring (CS) was used as standard.

Figure 3. Diagrammatic representation of the Wx-B1 protein sequences from the Spanish spelt types together with common wheat.

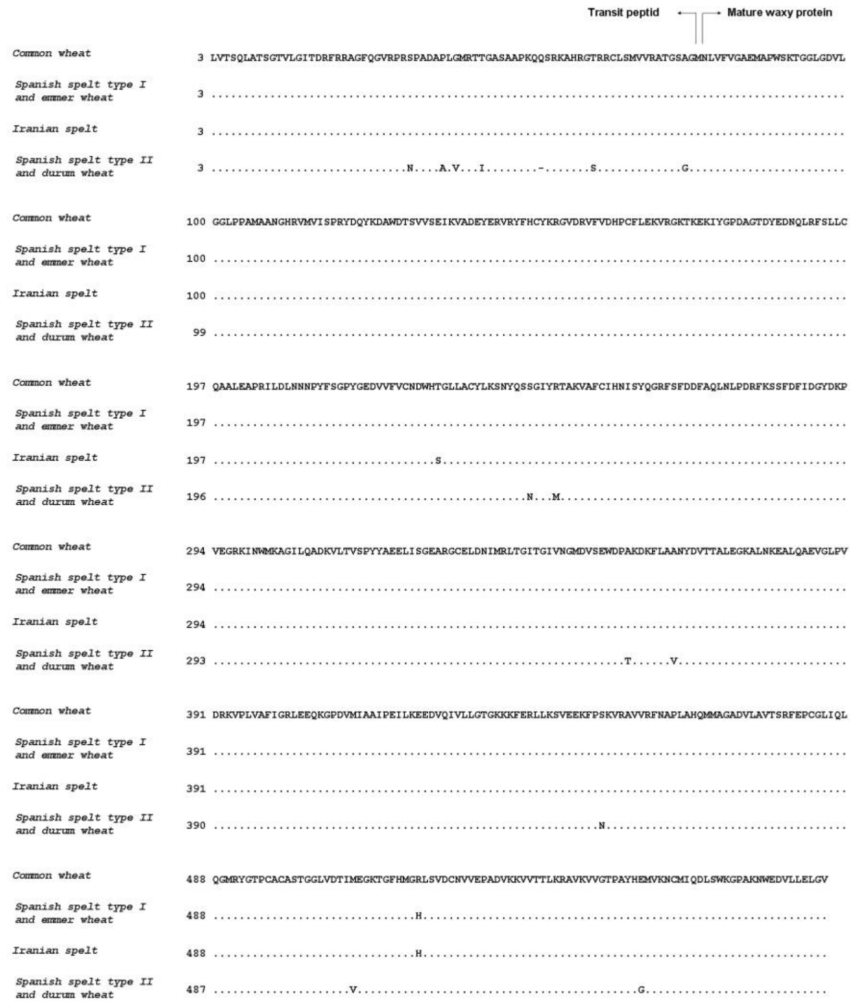

Figure 4. Neighbour-joining tree based on Maximum Composite likelihood method of *Wx* gene sequences in the evaluated wheat lines (bold), together with other previous sequences. Numbers in nodes indicated bootstrap estimates from 1000 replications.

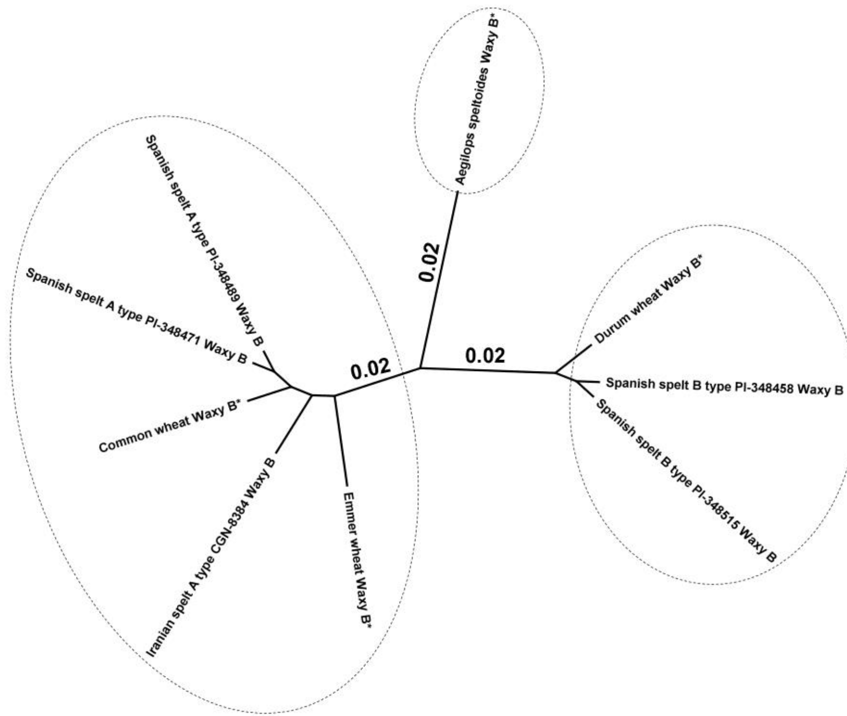

Table 1. Lines of wheat used in this study.

Specie	Ploidy	Genome	Cultivar/Accession[a]	Origin	Gene and GenBank No[b]
Triticum monococcum ssp. *monococum*	2×	A^mA^m	AUS22986	Australia	*Wx-A^m1:* AF110373
Triticum urartu	2×	A^uA^u	MG26992	Iraq	*Wx-A^u1: JN857937*
Aegilops speltoides	2×	S^sS^s	AUS21638	Iraq	*Wx-S^s1:* AF110374
Aegilops tauschii	2×	DD	CPI110799	-	*Wx-D1:* AF110375
Triticum turgidum ssp. *dicoccoides*	4×	AABB	-	-	*Wx-A1:* AB029061; *Wx-B1:* AB029062
Triticum turgidum ssp. *dicoccum*	4×	AABB	CGN16104	Germany	*Wx-A1:* **JN935600**; *Wx-B1:* **JN935601**
			PI275996	Spain	*Wx-A1: HM751941*; *Wx-B1: GQ205418*
			BGE012302	Spain	*Wx-A1: HM751941*; *Wx-B1: GQ205417*
Triticum turgidum ssp. *durum*	4×	AABB	Langdon	USA	*Wx-A1:* AB029063; *Wx-B1:* AB029064
			Mexicali	Mexico	*Wx-B1: GQ205420*
Triticum aestivum ssp. *aestivum*	6×	AABBDD	Chinese Spring	China	*Wx-A1:* AB019622; *Wx-B1:* AB019623; *Wx-D1:* AB019624
Triticum aestivum ssp. *spelta*	6×	AABBDD	CGN8384	Iran	*Wx-A1:* **HQ338720**; *Wx-B1:* **HQ338721**; *Wx-D1:* **HQ338722**
			CGN11460	Czech Rep.	*Wx-A1:* **JN935596**; *Wx-B1:* **JN935595**; *Wx-D1:* **JN935594**
			CGN11461	Germany	*Wx-A1:* **JN935598**; *Wx-B1:* **JN935599**; *Wx-D1:* **JN935597**
			CGN12269	Iran	*Wx-A1:* **JN935591**; *Wx-B1:* **JN935593**; *Wx-D1:* **JN935592**
			PI348458	Spain	*Wx-A1:* **HQ338723**; *Wx-B1:* **HQ338724**; *Wx-D1:* **HQ338725**

	PI348471	Spain	*Wx-A1:* **HQ338714***; Wx-B1:* **HQ338715***; Wx-D1:* **HQ338716**
	PI348489	Spain	*Wx-A1:* **HQ338717***; Wx-B1:* **HQ338718***; Wx-D1:* **HQ338719**
	PI348515	Spain	*Wx-A1:* **HQ338726***; Wx-B1:* **HQ338727***; Wx-D1:* **HQ338728**

[a] BGE: Centro de Recursos Fitogenéticos-INIA (Alcalá de Henares, Spain); CGN: Center for Genetic Resources (Wageningen, Netherlands); and PI: National Small Grain Collection (Aberdeen, USA).

[b] Bold text, sequenced in this study. Italic test, sequenced in our laboratory in previous works. The rest was obtained from GenBank.

Table 2. Summary of DNA polymorphism and test statistics for selection of the 38 sequences from polyploid wheat evaluated.

		Total						Coding region					
Gene	*n*	*k*	*s*	*h*	$\theta \times 10^{-3}$	$\pi \times 10^{-3}$	*D*	*k*	*s*	*h*	$\theta \times 10^{-3}$	$\pi \times 10^{-3}$	*D*
Wx-A1	14	5.67	22	7	2.6	2.1	-0.7792 ns	3.93	15	7	2.7	2.8	-0.6767 ns
Wx-B1	15	54.40	163	10	18.9	20.5	0.3760 ns	17.68	53	10	9.5	10.3	0.3643 ns
*Wx-B1*I	12	5.6	22	8	2.7	2	-1.0188 ns	4.12	15	8	2.8	2.3	-0.7311 ns
*Wx-B1*II	3	6.66	10	2	2.4	2.4	-	3.33	5	2	1.9	1.9	-
Wx-D1	9	1.56	4	4	0.5	0.6	0.2315 ns	1.11	2	2	0.4	0.6	1.7542 ns
Mean					7.3	7.7					4.2	4.6	

n: number of sequences; *k*: average number of nucleotide differences; *s*: number of polymorphic sites; *h*: number of haplotypes; θ: Watterson's estimate; π: nucleotide diversity; and *D:* Tajima's estimate *D*-test. ns: not significant. The total length of the sequences was 2695-2690 bp for *Wx-A1,* 2702 for *Wx-B1* type I, 2717 for *Wx-BI* type II and 2771 for *Wx-D1*; the length of the coding regions analysed was of 1724 bp except for *Wx-B1* type I that was 1727.

Table 3. Assignation of the allelic variation for *Wx* genes according with the data obtained in the current study.

Gene	Putative allele	Cultivar/Accession	According to sequence	
			DNA	Protein
Wx-A1	*a*	Common wheat: Chinese Spring	1	1
		Wild emmer	2	2
		Durum wheat: Langdon	3	3
		Emmer: PI275996; Emmer: BGE012302	4	
		Spelt: CGN8384; Spelt: CGN11460; Spelt: CGN11461; Spelt: PI348458; Spelt: PI348471	5	
		Spelt: PI348489; Spelt: PI348515	6	
		Emmer CGN16104; Spelt: CGN12269	7	4
Wx-B1	*a*	Common wheat: Chinese Spring	1	1
		Wild emmer	2	2
		Durum wheat: Langdon	3	3
		Spelt: PI348458; Spelt: PI348515	4	
		Spelt: CGN8384	5	4
		Spelt: CGN11460; Spelt: CGN11461; Spelt: CGN12269;	6	7
		Emmer: CGN16104; Spelt: PI348471; Spelt: PI348489;	7	
	*c**	Emmer: PI275996	8	5
	c'	Durum wheat: Mexicali	9	6
	g	Emmer: BGE012302	10	7
Wx-D1	*a*	Common wheat: Chinese Spring	1	1
		Spelt: CGN11460; Spelt: CGN11461; Spelt: CGN12269;	2	
		Spelt: PI348458;	3	
		Spelt: CGN8384; Spelt: PI348471; Spelt: PI348489; Spelt: PI348515	4	

Table 4. Variation between homoeologous and orthologous *Wx* genes and estimated divergence times between them.

Gene pairs	*Ks*	*Ka*	*Ka/Ks*	MYA*
Wx-A1 vs. *Wx-D1*	0.144	0.017	0.121	2.7
Wx-A1 vs. *Wx-B1*	0.123	0.021	0.173	2.3
Wx-B1 vs. *Wx-D1*	0.127	0.015	0.116	2.4
Wx-B1 (type I) vs. *Wx-B1* (type II)	0.032	0.005	0.142	0.6

* Divergence rate of 0.0533 synonymous substitution per MY calculated according with Dvorak and Akhunov (2005).

1

2