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

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• *Background and Aims* Waxy proteins are responsible for amylose synthesis in wheat seeds, being encoded by three *waxy* genes (*Wx-A1*, *Wx-B1* and *Wx-D1*) in hexaploid wheat. In addition to their role in starch quality, *waxy* loci have been used to study the phylogeny of wheat. The origin of European spelt (*Triticum aestivum* ssp. *spelta*) is not clear. This study compared *waxy* gene sequences of a Spanish spelt collection with their homologous genes in emmer (*T. turgidum* ssp. *dicoccum*), durum (*T. turgidum* ssp. *durum*) and common wheat (*T. aestivum* ssp. *aestivum*), together with other Asian and European spelt that could be used to determine the origin of European spelt.
• *Methods waxy* genes were amplified and sequenced. Geneious Pro software, DNAsp and MEGA5 were used for sequence, nucleotide diversity and phylogenetic analysis, respectively.
• *Key Results* Three, four and three new alleles were described for the *Wx-A1*, *Wx-B1* and *Wx-D1* loci, respectively. Spelt accessions were classified into two groups based on the variation in *Wx-B1*, which suggests that there were two different origins for the emmer wheat that has been found to be part of the spelt genetic make-up. One of these groups was only detected in Iberian material. No differences were found between the rest of the European spelt and the Asiatic spelt, which suggested that the Iberian material had a different origin from the other spelt sources.
• *Conclusions* The results suggested that the *waxy* gene variability present in wheat is undervalued. The evaluation of this variability has permitted the detection of ten new *waxy* alleles that could affect starch quality and thus could be used in modern wheat breeding. In addition, 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.

Key words: Wheat, *Triticum aestivum* ssp. *spelta*, 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 ten introns, as Murai *et al.* (1999) showed on 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 basis 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.*, 2008*a*; 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*; $2n = 6x = 42$, AABBDD), currently a 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, 2004*a*, *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., 2008*b*). Another important aspect, the starch quality, has also been 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) and a cultivated hexaploid wheat, probably *T. aestivum* L. ssp. *compactum* Host em. Mackey (Liu and Tsunewaki, 1991; Yan *et al.*, 2003; Blatter *et al.*, 2004; Dedkova *et al.*, 2004). More recently, Dvorak *et al.* (2012) showed that some forms of the Asiatic spelt could also have their origin in free-threshing wheat. In addition to this, other workers (Dedkova *et al.*, 2004; Elia *et al.*, 2004) have suggested that European spelt could be classified into two eco-geographical groups: the Iberian (pol. *ibericum* Flaskb.) and the Bavarian (pol. *bavaricum* Vav.) geographical groups. These authors suggested that Iberian spelt could have its origin in Asia and be different from the other types of European spelt. *Waxy* gene sequences have been utilized to study the origin and phylogeny of other *Poaceae* species, including wheat (Mason-Gamer *et al.*, 1998; Yan *et al.*, 2000, 2003; Mason-Gamer, 2001; Yan and Bhave, 2001; Ingram and Doyle, 2003; Fortune *et al.*, 2007), and, consequently, the characterization of the *waxy* genes in this Spanish spelt collection could shed light on this question about the origin of Spanish spelt.

The aim of the current study was the molecular characterization of the *waxy* genes present in this Spanish spelt collection, and to compare them with their homologous genes in emmer, durum and common wheat, together with other spelt examples of Asian and European origin that could be used for phylogenetic studies on the origin of European spelt.

MATERIALS AND METHODS

Plant material

Six Spanish and two Iranian spelt [*Triticum aestivum* ssp. *spelta* L. em. Thell.; also considered as *Triticum* sect. *Spelta* (Wolf) Dumort.] accessions obtained from three Germplasm Banks were analysed first for their waxy protein composition. The six Spanish accessions (PI348458, PI348471, PI348489, PI348515, PI348595 and PI3487447) were from the National Small Grain Collection (Aberdeen, ID, USA) while the Iranian accessions (CGN8384 and CGN12269) were obtained from the Center for Genetic Resources (The Netherlands). One further spelt accession (CGN11460, Czech Republic origin) and one emmer German accession (CGN16104) were also used 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 bisacrylamide concentration (approx. 0·44 %).

DNA extraction and PCR amplification

For DNA extraction, approx. 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, Carlsbad, CA, USA).

The primers designed by Monari *et al.* (2005) were used to amplify the central region (the region spanning from the middle of exon 3 to exon 6) of the *waxy* genes: WxBAF (5'-ACTTCCACTGCTACAGCGCGGGGT-3') and WxBAR (5'-GCTGACGTCCATGCCGTTGACGATG-3'). Each 15 μL reaction included 50 ng of DNA, 1·5 mM $MgCl_2$, 0·2 μM of each primer, 0·2 mM dNTPs, 1·5 μL of 10× PCR buffer and 0·75 U of 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.

Amplificied and digested products were fractionated in vertical polyacrylamide gels with an 8 % polyacrylamide concentration (w/v, approx. 1·28 %), and the bands were visualized by ethidium bromide staining.

Cloning of waxy genes and sequencing analysis

To clone almost the entire sequence of the *waxy* genes, besides primers WxBAF and WxBAR described above, pairs of primers WxF3 (5'-TCTGGTCACGTCCCAGCTCGCCACCT-3')/WxVT1R (5'-ACCCCGCGCTTGTAGCAGTGGAAGT-3') ($T_m = 64$ °C) and WxVT1F (5'-CATCGTCAACGGCATGGACGTTCAGC-3')/WxVTR (5'-CCAGAAGCACGTCCTCCCAGTTCTTG-3') ($T_m = 64$ °C) were used to amplify the beginning and the end of the *waxy* genes, respectively. PCR products were excised from the polyacrylamide gel and cloned into pGEM T-easy vector (Promega, Madison, WI, USA) for sequencing. Inserts were sequenced from at least three different clones using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA).

The sequences reported in the current study were compared with the sequences available in the GenBank database using Geneious Pro ver. 5·0·3 software (Biomatters Ltd).

Data analysis

DNA analyses were conducted by DNAsp ver. 5·0 (Librado and Rozas, 2009). Nucleotide diversity was estimated as θ, the number of segregating (polymorphic) sites (Watterson, 1975), and 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 relationship *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) between the A and D genome estimated by Dvorak and Akhunov (2005).

The phylogenetic tree was constructed with the software MEGA5 (Tamura *et al.*, 2011) using the complete coding regions. A Neighbor–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 one-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 pattern of waxy proteins (Fig. 1A, lanes 3–4). This similarity was also observed when the samples were separated by 2-D isoelectic focusing × SDS–PAGE separation (Fig. 1B, C).

However, the comparison of these supposedly wild alleles by PCR amplification of genomic DNA and posterior electrophoretic migration showed two groups within the genotypes analysed. The most important differences between the samples analysed were detected when the central region of the *waxy* genes was amplified (Fig. 2A). In this figure, the samples used were the same as those shown in Fig. 1, and appear in the same order. Two Spanish and two Iranian spelt samples showed three bands (Fig. 2A, lanes 1–4), each one corresponding to one of the three identified *waxy* genes, of the expected size of: *Wx-D1* (1017 bp), *Wx-A1* (958 bp) and *Wx-B1* (935 bp), based on the published sequences of *Wx-A1a*, *Wx-B1a* and *Wx-D1a* alleles of cv. Chinese Spring (Murai *et al.*, 1999). This fact meant that they were similar to the common wheat (cv. Chinese Spring; Fig. 2A, lanes 1–3, 4 and 5, respectively). However, the other spelt samples (Fig. 2A, lanes 5–8) had only two bands, as the *Wx-A1* and *Wx-B1* bands had co-migrated. The first group was called spelt type I and the second spelt type II.

To clarify these band patterns and to confirm the presence of the two spelt groups, the PCR products were digested using the endonuclease, *Bgl*I. Based on the published sequences for common wheat (cv. Chinese Spring), only one sequence in the *Wx-B1* gene was targeted. Two bands of 897 and 38 bp were expected from the digestion of the *Wx-B1* spelt I gene. While the 897 bp band was found in all samples, the 38 bp band was lost due to its small size (Fig. 2B). In the spelt II lines, one additional band was separated from the *Wx-A1* band after the digestion (Fig. 2B, lanes 5–8), confirming the presence of a larger *Wx-B1* band that had co-migrated with the *Wx-A1* band shown in Fig. 2A.

Sequences of spelt waxy genes and deduced proteins

In order to characterize the variation found by electrophoretic methods, the three *waxy* genes from each Spanish spelt type (I and II) and the two Iranian samples were sequenced. *Waxy* genes from one European spelt accession and one emmer accession were also sequenced. All these sequences were uploaded to the GenBank database (Table 1). Comparisons between these sequences and others from emmer, durum and common wheat previously available in GenBank were undertaken. The criteria used to choose these materials was to compare potential material with the different origins of the spelt (Asian and European) and the putative progenitors that may be involved in the different crossing events that generated spelt (emmer and common wheat).

A summary of DNA polymorphism found in the *Wx* sequences evaluated is shown in Table 2. These data were evaluated using both the complete sequence (exons + introns) and just the coding sequence (exons). In both cases, the *Wx-B1* gene displayed the highest level of single nucleotide polymorphisms (SNPs) among the three genes, with 163 polymorphic sites in the complete sequence and 53 in the coding region (Table 2). In the latter, 36 of these nucleotide substitutions were synonymous (silent mutations) while the rest were non-synonymous, which implied changes in the amino acid sequences. The ten alleles identified encoded seven different polypeptides (Table 3). This polymorphism was clear lower down in the coding regions of the other two genes (Tables 2 and 3). The *Wx-A1* gene coding region showed 15 nucleotide substitutions (eight synonymous and seven non-synonymous), while the *Wx-D1* gene only presented two substitutions that were synonymous. Consequently, only four and one polypeptides were obtained for the seven and two alleles detected in the coding regions of the *Wx-A1* and *Wx-D1* genes, respectively (Table 3).

Two statistics, π (Nei, 1987) and θ (Watterson, 1975), were used to estimate nucleotide diversity (Table 2). Both values were similar in all the cases that were associated with a drift–mutation balance. The values for the *Wx-B1* gene were higher than in the other two genes. Tajima's *D* values were not significant for the three genes, which was consistent with a neutral equilibrium (Table 2). The value was negative for the *Wx-A1* gene, indicative of an excess of low frequency alleles, while the other two genes showed positive values, which indicated an excess of intermediate frequency alleles.

The data shown in Table 3 suggested that the previous classification of *waxy* genes in Spanish spelt was incorrect. In all cases, only the class 1 DNA and protein sequences corresponded to the wild allele for each locus. For the *Wx-A1* gene, six classes, previously classified as *Wx-A1a*, could be

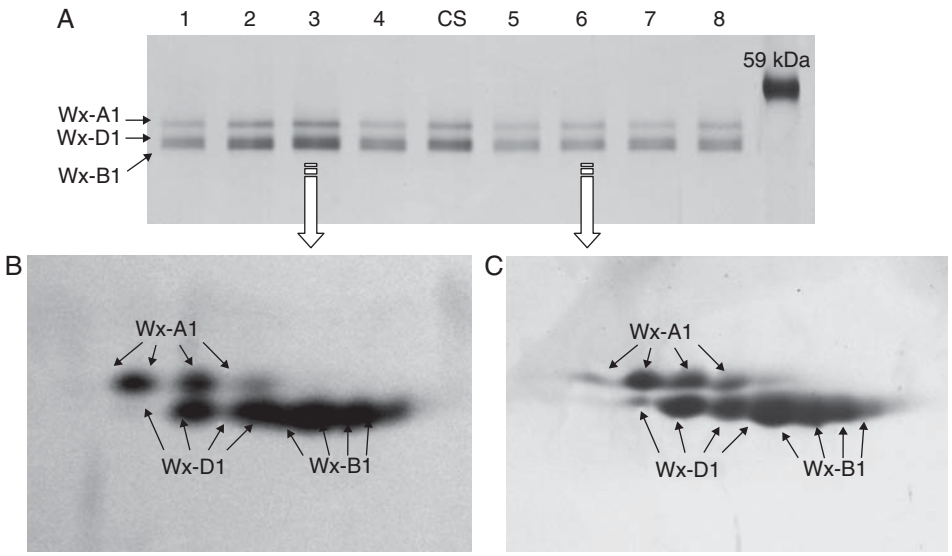

FIG. 1. SDS–PAGE patterns of waxy proteins. (A) One-dimensional; and (B) two-dimensional separation. Lanes are as follows: 1, PI348471; 2, PI348489; 3, CGN12269; 4, CGN8384; 5, PI348515; 6, PI348458; 7, PI348747; and 8, PI348595. The cv. Chinese Spring (CS) was used as a standard.

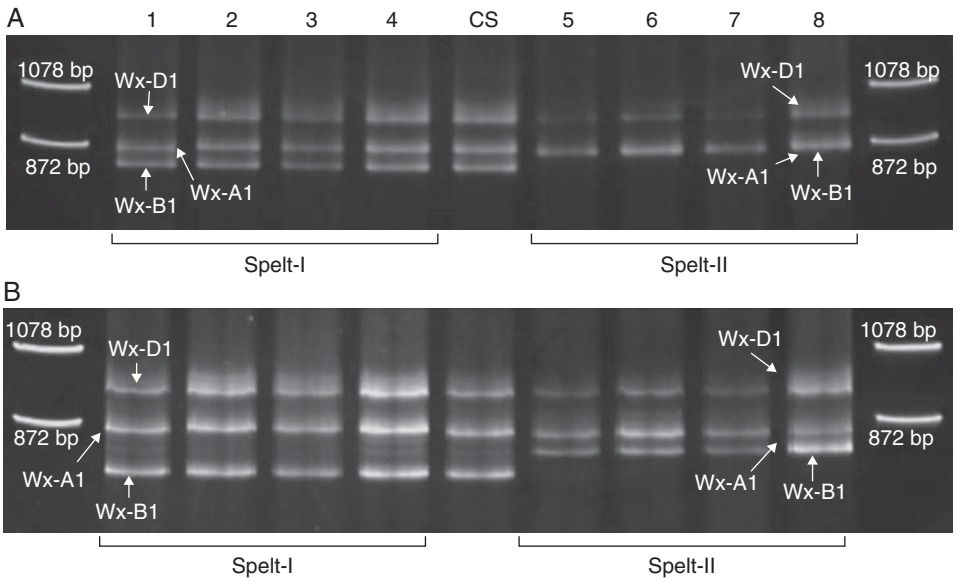

FIG. 2. (A) Amplification products of the central region of the *waxy* genes; and (B) amplification products of the central regions of the *waxy* genes digested with *Bgl*I. Arrowheads indicates products of the digestion. Lanes are as follows: 1, PI348471; 2, PI348489; 3, CGN12269; 4, CGN8384; 5, PI348515; 6, PI348458; 7, PI348747; and 8, PI348595. The cv. Chinese Spring (CS) was used as a standard.

reclassified because the nucleotide sequences were different from those in the *Wx-A1a* allele present in common wheat. However, four of them synthesized a polypeptide with the same amino acid sequence, which included all the Spanish spelt types evaluated (Table 3).

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

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

TABLE 1. *Lines of wheat used in this study*

Species	Ploidy	Genome	Cultivar/ accession*	Origin	Gene and GenBank no.†
Triticum monococcum ssp. *monococum*	2*x*	A^mA^m	AUS22986	Australia	*Wx-A^{m}1*: AF110373
Triticum urartu	2*x*	A^uA^u	MG26992	Iraq	*Wx-A^{u}1*: *JN857937*
Aegilops speltoides	2*x*	S^sS^s	AUS21638	Iraq	*Wx-S^{s}1*: AF110374
Aegilops tauschii	2*x*	DD	CPI110799	–	*Wx-D1*: AF110375
Triticum turgidum ssp. *dicoccoides*	4*x*	AABB	–	–	*Wx-A1*: AB029061; *Wx-B1*: AB029062
Triticum turgidum ssp. *dicoccum*	4*x*	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*x*	AABB	Langdon	USA	*Wx-A1*: AB029063; *Wx-B1*: AB029064
			Mexicali	Mexico	*Wx-B1*: *GQ205420*
Triticum aestivum ssp. *aestivum*	6*x*	AABBDD	Chinese Spring	China	*Wx-A1*: AB019622; *Wx-B1*: AB019623; *Wx-D1*: AB019624
Triticum aestivum ssp. *spelta*	6*x*	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**

* BGE, Centro de Recursos Fitogenéticos-INIA (Alcalá de Henares, Spain); CGN, Center for Genetic Resources (Wageningen, The Netherlands); PI, National Small Grain Collection (Aberdeen, ID, USA).

† Bold text, sequenced in this study; italic text, sequenced in our laboratory in previous works. The remaining accession numbers were 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	−0·779 ns	3·93	15	7	2·7	2·8	−0·677 ns
Wx-B1	15	54·40	163	10	18·9	20·5	0·376 ns	17·68	53	10	9·5	10·3	0·364 ns
Wx-B1 (type I)	12	5·6	22	8	2·7	2	−1·019 ns	4·12	15	8	2·8	2·3	−0·731 ns
Wx-B1 (type 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	0·232 ns	1·11	2	2	0·4	0·6	1·754 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; *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 1724 bp, except for *Wx-B1* type I that was 1727 bp.

the *Wx-A1* gene compared with 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 with spelt type I or with common wheat. Specifically, the deduced Wx-A1 protein from the two spelt types was similar and showed three amino acid differences compared with the Wx-A1 protein from common wheat (*Wx-A1a* allele): Phe60 → Gly, Asp61 → Asn and Trp454 → 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 (Glu333 → Gly and Trp454 → 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: Met42 → Ile and Ser75 → 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.

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	

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 both types of polypeptides were notable (Fig. 3): Ser34 → Asn, Pro39→ Ala, Gly41 → Val, Thr45 → Ile, Gln54 → –, Thr62 → Ser, Ala76 → Gly, Ser246 → Asn, Arg250 → Met, Ala358 → Thr, Ala365 → Val, Ser451 → Asn, Met510 → Val and Glu554 → Gly. In a previous study based on protein analysis (Yamamori *et al.*, 1995), the *Wx* allele present in cv. Langdon was classified as *Wx-B1a*, probably due to a similar problem to the one that arose with the Spanish spelt in the current study. However, the current analysis showed that both alleles were very different. Spelt type I lines showed only one difference with respect to common wheat: Arg520 → His.

In summary, analyses of the Spanish spelt lines that were previously classified as wild genotypes (*Wx-A1a*, *Wx-B1a* and *Wx-D1a*) have shown that, although they presented one unique allele for the *Wx-A1* gene, this was not the *Wx-A1a* allele. Furthermore, the Spanish spelt lines showed two different alleles for the *Wx-B1* gene, neither of which was the *Wx-B1a* allele.

Phylogenetic analysis

A phenogram based on the Maximum Composite Likelihood method was constructed using all the *waxy* sequences evaluated in this study, together with the putative donors from the genomes present in tetra- and hexaploid wheats (Fig. 4). *Triticum urartu* Thum. ex Gandil (A^uA^u, *Wx-*A^u*1*: JN857937) and einkorn (*T. monococcum* L. ssp. *monococum*; A^mA^m, *Wx-*A^m*1*: AF110373) were included from the A genome; *Aegilops speltoides* Tausch. (S^sS^s, *Wx-*S^s*1*: AF110374) was included from the B genome and *A. tauschii* Coss. (DD, *Wx-D1*: AF110375) was included from the D genome. The total nucleotide sequence (exons + introns) was used in all cases.

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

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

The *Ks* and *Ka* substitution rates among *Wx* genes both for homeologous (*Wx-A1*, *Wx-B1* and *Wx-D1*) as for orthologous (*Wx-B1* type I and *Wx-B1* type II) genes were calculated by using the coding sequence of the complete gene. The comparison values of the homeologous genes were approx. 3-fold higher than the value obtained for the two types of *Wx-B1* genes (Table 4). Consequently, the divergence time between both *Wx-B1* gene types was estimated as approx. 0·6 million

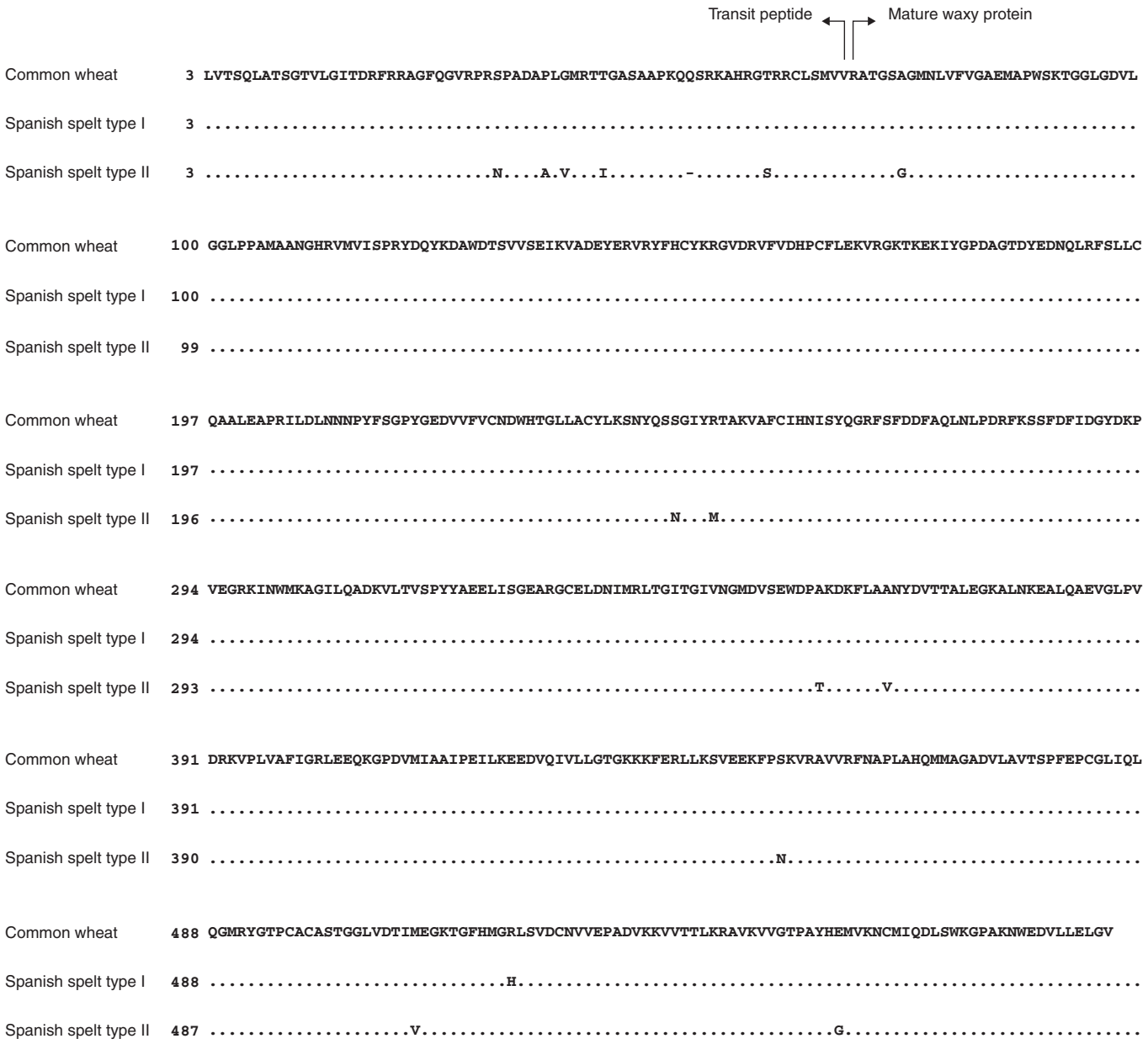

FIG. 3. Diagrammatic representation of the Wx-B1 protein sequences from the Spanish spelt types together with common wheat.

years ago (Table 4), based on the mean divergence time for the separation between the A and D genome (2·7 million years ago) estimated by Dvorak and Akhunov (2005).

DISCUSSION

The major nutritional component in wheat grains is starch, which is formed by two glucose polymers: amylose and amylopectin, whose synthesis involves up to five starch synthases (Baldwin, 2001). The variation in the ratio between both polymers, 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;

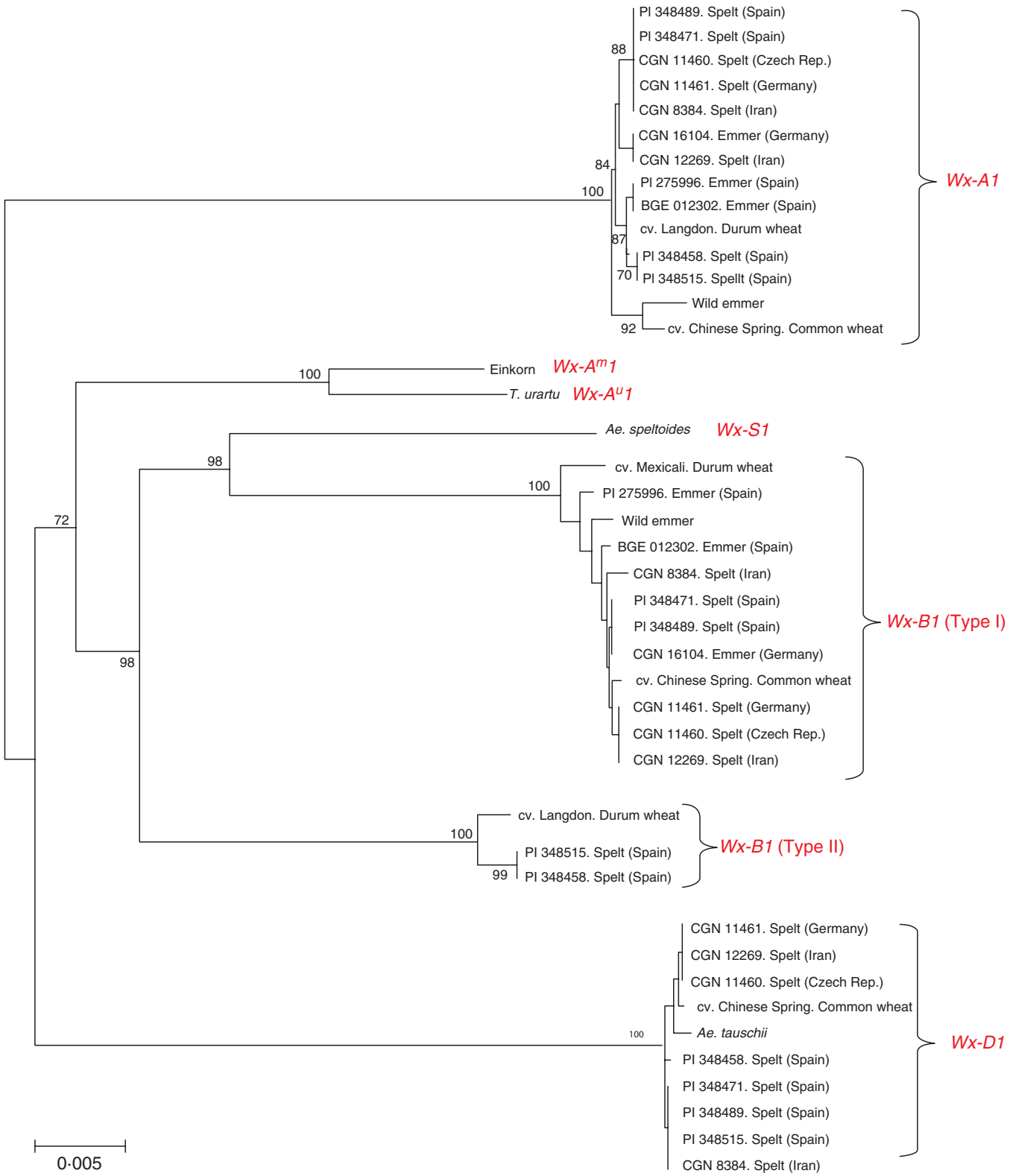

FIG. 4. Neighbor-joining tree based on the maximum composite likelihood method of *Wx* gene sequences in the evaluated wheat lines (bold), together with other previous sequences. Numbers in nodes indicate bootstrap estimates from 1000 replications.

Yanagisawa *et al.*, 2001). However, the characterization of the apparently functional enzymes has been less well 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

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 substitutions per million years calculated according to Dvorak and Akhunov (2005).

that had not shown any differences when they were previously evaluated for waxy protein polymorphism by SDS–PAGE (Guzman *et al.*, 2010).

Although other studies have found high levels of nucleotide diversity for these genes in common wheat (Huang and Bûlé-Babel, 2012), it is important to emphasize 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 synthesized proteins. However, the variation found in the nucleotide sequences was considerably higher. Seven, ten and four different alleles for the *Wx-A1*, *Wx-B1* and *Wx-D1* genes, respectively, were detected where only one polypeptide was detected by SDS–PAGE for each gene. This variation at the DNA level was finally confirmed by the existence of three and six novel *waxy* alleles for the Wx-A1 and Wx-B1 proteins, respectively. The new alleles detected could have a different functionality fromn that of the wild-type alleles and thus affect the composition (amylose/amylopectin) and functionality of starch. This fact should be tested in further experiments transferring these alleles to the same genetic background and evaluating their use for modern wheat breeding.

In addition to the potential improvements that these mutations could provide in starch quality, variation in the nucleotide sequence of the *Wx* genes has been identified as a useful tool in phylogenetic analysis (Mason-Gamer *et al.*, 1998, Yan *et al.*, 2000; Mason-Gamer, 2001; Ingram and Doyle, 2003; Fortune *et al.*, 2007). The *Wx-A1*, *Wx-B1* and *Wx-D1* spelt sequences were compared with the homologous genes in the putative donors of A, B and D genomes in polyploid wheats to shed more light on the origin of each of the genomes. The current data only supported the hypothesis that suggested that *A. tauschii* is the D genome donor (Dvorak *et al.*, 1998), whereas there were no data to support the second hypothesis about the origin of the A and B genomes. The current theory on the origin of the A genome in hexaploid wheat suggests that the wild wheat species (*T. urartu*) could be the A genome donor (Dvorak *et al.*, 1993). However, the *Wx-A^{u}1* sequence obtained in a previous study (Guzman and Alvarez, 2012) was clearly different from the rest of the *Wx-A1* sequences (Fig. 4), with the exception of the *Wx-A^{m}1* gene sequenced in einkorn by Yan *et al.* (2000). Similar results were found when the homology between the *Wx-S1* gene from *A. speltoides*, the main candidate for the B genome (Petersen *et al.*, 2006), and the *Wx-B1* gene were evaluated. Although these results should be treated with caution, due to the fact that only one sequence of *A. speltoides* was used, these data suggested that the homology among these sequences was low and this proposed origin was not clear based on the information derived from *waxy* genes.

However, it is remarkable that the *Wx-B1* genes from the polyploid wheats evaluated in this study clearly separated into two different groups. The *Wx-B1* type I group had a greater similarity to the *Wx-S1* gene than the type II group. Although the type II group had been associated with one durum wheat cultivar (cv. Langdon), the genealogy of this cultivar [Langdon = Carleton/Mindum//Khapli/3/Carleton/Mindum/Stewart/4/Stewart] by Zeven and Zeven-Hissink (1976) indicated that it had been generated using several lines of emmer wheat (Carleton = vernal emmer/Mindum, Khapli = emmer and Stewart = Mindum/2* vernal emmer). This suggested that there were two different origins for the emmer wheat that has been found to be part of the spelt genetic make-up, although, to date, the second origin has only been found in the Spanish spelt. These results confirm the unique nature of the Iberian spelt gene pool indicated by other studies (Dedkova *et al.*, 2004; Elia *et al.*, 2004), who suggested that Iberian spelt (pol. *ibericum* Flaskb.) could have its origin in Asia and be different from the rest of the European spelts, which are said to derive from a secondary hybridization between emmer and a cultivated hexaploid wheat, probably club wheat, as several workers have proposed (Liu and Tsunewaki, 1991; Yan *et al.*, 2003; Blatter *et al.*, 2004; Dedkova *et al.*, 2004). This last hypothesis has not been totally verified (for a review, see Salamini *et al.*, 2002) as the current data have not revealed major differences between the *Wx* sequences for the three genomes of European and Asian spelt evaluated. In the same way, a recent hypothesis exposed by Dvorak *et al.* (2012) that suggested that all spelt (except the 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 of the accessions. In addition to this, we also found two different groups based on B genome differences, a fact that could disagree with their hypothesis. The same authors also suggested that the hexaploid ancestor could have its origin in the cross of *A. tauschii* with a free-threshing tetraploid, and not emmer wheat. This fact could also be revised in further surveys with *waxy* data from more tetraploid species, such as durum wheat. On the other hand, other studies have estimated that the time of the origin of tetraploid wheat could be between 0·36 and 0·50 million years ago (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 could have emerged during the synthesis of the tetraploid wheat. Although this hypothesis would be confirmed with further studies, this opens up the possibility that the origin of these wheats could be a 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 and

Aegilops sp.) in order to clarify further 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 underestimated because some of these variants were not detected by the traditional classification based on 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.

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