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Wx Gene in *Hordeum chilense:* Chromosomal Location and Characterisation of the Allelic Variation in the Two Main Ecotypes of the Species

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Received: 17 April 2019; Accepted: 17 May 2019; Published: 22 May 2019

Abstract: Starch, as the main grain component, has great importance in wheat quality, with the ratio between the two formed polymers, amylose and amylopectin, determining the starch properties. Granule-bound starch synthase I (GBSSI), or waxy protein, encoded by the *Wx* gene is the sole enzyme responsible for amylose synthesis. The current study evaluated the variability in *Wx* genes in two representative lines of *Hordeum chilense* Roem. et Schult., a wild barley species that was used in the development of tritordeum (×*Tritordeum* Ascherson et Graebner). Two novel alleles, *Wx-H*^{ch}1*a* and *Wx-H*^{ch}1*b*, were detected in this material. Molecular characterizations of these alleles revealed that the gene is more similar to the *Wx* gene of barley than that of wheat, which was confirmed by phylogenetic studies. However, the enzymatic function should be similar in all species, and, consequently, the variation present in *H. chilense* could be utilized in wheat breeding by using tritordeum as a bridge species.

Keywords: starch; tritordeum; waxy proteins; wheat quality; wild barley

1. Introduction

Starch is the main component of wheat grain, constituting up to 75% of its dry weight. This polysaccharide contains two different glucose polymers: amylose (22%–35% of the total) and amylopectin (68%–75% of the total) [1]. Changes in the ratio between these polymers have a clear influence on starch gelatinization, pasting and gelation properties [2], affecting the end-use quality levels of different wheat products, such as bread, pasta, and noodles [3–5], as well their shelf-lives [6] and nutritional values [7].

Starch synthesis involves several starch synthases, starch branching enzymes, and starch debranching enzymes [8]. The most studied of these proteins has been the granule-bound starch synthase I (GBSSI) or waxy proteins (ADP glucose starch glycosyl transferase, EC 2.4.1.21), which are solely responsible for amylose synthesis [9]. In wheat, these proteins are synthesized by genes located in the short arm of the seven-group homeologous chromosome, with the exception of the *Wx-B*1 gene that, owing to a translocation event, is located in the 4AL chromosome [10]. In wheat relatives, this gene is located in similar positions, and its molecular configuration of 12 exons and 11 introns is highly conserved in all of these species [11]. In other Poaceae species, such as barley (*Hordeum vulgare* L.), this gene has shown the identical structure and location [12].

The variability of waxy proteins has been studied in common and durum wheat, as well as in some wild and cultivated relatives [13]. However, the variability in modern wheat cultivars is not very wide, according to data in the Wheat Gene Catalogue [14]. In the search for new waxy variants, species

from the primary and secondary wheat pools could contain good candidates. These species have been successfully used to transfer useful traits to wheat. In some cases, these transfer events have generated amphiploids that have also been used as bridge species [15]. In other cases, these amphiploids have been derived to produce human-made crops, such as tritordeum (×*Tritordeum* Ascherson et Graebner), that have shown promising characteristics [16].

Tritordeum was synthesized using mainly durum wheat and *Hordeum chilense* Roem. et Schult. ($2n = 2\times = 14$, $H^{ch}H^{ch}$), a wild barley species native to Chile and Argentina, included in the section *Anisolepis* Nevski [17]. In this species, variation in genes related to quality has been widely evaluated over the last decade [18–21] and has been used to expand the genetic base of tritordeum. Furthermore, this species exhibits advantageous agronomic and quality characteristics [22–24], which, together with its ability to be crossed with other members of the *Triticeae* tribe [25], make it useful in cereal breeding.

In its natural distribution area, *H. chilense* shows some ecotypes, based on morphological and ecophysiological traits [26,27]. The two main groups are related to the first two *H. chilense* lines used to develop tritordeum, H1 and H7. Tritordeum developed using these lines showed differences in fertility, grain size, and life cycle, depending on the female parent (H1 or H7) used [25]. An analysis of the genes related to the flour quality from both lines also showed that these ecotypes could have different effects on the tritordeum quality. These differences have been evaluated for the seed storage proteins [18,28], hordoindolines [29], and pigment enzymes [30,31].

The main goals of this study were to analyze allelic variation and molecularly characterize of the *Wx* genes in the H1 and H7 lines of *H. chilense*, and to determine the gene's chromosomal location.

2. Materials and Methods

2.1. Plant Materials

Seeds of two *H. chilense* lines (H1 and H7) that were self-pollinated for two generations were used in this study. The ditelosomic addition lines (CS + $7H^{ch}S$ and CS + $7H^{ch}L$), together with both parental lines (common wheat cv. "Chinese Spring" and line H1) were used to locate the *Wx* gene in *H. chilense*. These materials were grown in greenhouse conditions.

2.2. DNA Extraction and PCR Amplification

For DNA extractions, ~100 mg of young leaf tissue was excised and immediately frozen in liquid nitrogen. DNA was isolated using the cetyltrimethyl ammonium bromide (CTAB) method as described by Stacey and Isaac [32].

The primers BDFL (5′-CTGGCCTGCTACCTCAAGAGCAACT-3′) and BRD (5′-CTGACGTCCATGCCGTTGACGA-3′) designed by Nakamura et al. [33] were used to detect the presence of the *Wx-*H^{ch}*1* gene in the ditelosomic addition lines. The amplification was performed in a 20 µL final reaction volume, containing 50 ng of genomic DNA, 1.25 mM $MgCl_2$, 0.2 mM dNTPs, 4 µL 10× PCR buffer, 0.2 µM of each primer and 0.75 U GoTaq® G2 Flexi DNA polymerase (Promega). The PCR conditions included an initial denaturation step of 3 min at 94 °C followed by 35 cycles as follows: 30 s at 94 °C, 30 s at 65 °C then 2 min at 72 °C. After the 35 cycles, a final extension of 5 min at 72 °C was included.

Amplification products were fractionated in vertical PAGE gels with 8% polyacrylamide concentration (*w*/*v*, C: 1.28%) and the bands were stained with GelRed™ nucleic acid staining (Biotium) and visualized under UV light.

2.3. Cloning of PCR Products and Sequencing Analysis

Owing to the length and structure of the *Wx* gene, ~2800 bp with 11 introns and 12 exons, three fragments were amplified using primers designed by Guzmán and Alvarez [34]. The first fragment includes the first to third exons (Wx1Fw: 5′-TTGCTGCAGGTAGCCACACC-3′ and Wx1Rv: 5′-CCGCGCTTGTAGCAGTGGAA-3′), the second extends from the third to the sixth exon

(Wx2Fw: 5′-ATGGTCATCTCCCCGCGCTA-3′ and Wx2Rv: 5′-GTTGACGGCGAGGAACTTGT-3′), and the last fragment covers the region spanning the 6th to the 11th exon (Wx3Fw: 5′-GGCATCGTCAACGGCATGGA-3′ and Wx3Rv: 5′-TTCTCTCTTCAGGGAGCGGC-3′).

All amplifications were performed in 50 µL final volumes, containing 100 ng of DNA genomic, 1.25 mM $MgCl_2$, 0.2 mM dNTPs, 10 µL 10× PCR buffer and 0.75 U GoTaq®G2 Flexi DNA polymerase (Promega). The primer concentrations were 0.4, 0.3 and 0.2 µM per primer for the first, second and third fragments, respectively. The PCR conditions included an initial denaturation step of 3 min at 94 °C and then 35 cycles as follows: for Wx1Fw/Wx1Rv, 40 s at 94 °C, 30 s at 64 °C and 1 min at 72 °C, for Wx2Fw/Wx2Rv, 30 s at 94 °C, 30 s at 66 °C and 90 s at 72 °C, and for *Wx3Fw*/*Wx3Rv*, 40 s at 94 °C, 30 s at 62 °C and 90s at 72 °C. After the 35 cycles, all reactions included a final extension of 5 min at 72 °C.

The PCR products were purified by separation in 1% agarose gel, excised and then independently ligated into the pSpark®-TA Done vector (Canvax). They were then transformed into *Escherichia coli* 'CVX5α' competent cells (Canvax). Inserts were sequenced by Sanger method from at least three different clones. The novel sequences are available from the GenBank database [*Wx-H*^{ch}*1a*: MK045501 for the H1 line, and *Wx-H*^{ch}*1b*: MK045502 for the H7 line].

2.4. Data Analysis

The sequences were analyzed and compared with sequences of CS (*Wx-A*1: AB019622, *Wx-B1*: AB019623, and *Wx-D1*: AB019624), two-rowed barley cv. Vogelsanger Gold (*Wx-H*1: X07931) and six-rowed barley cv. Morex (*Wx-H1*: AF474373) available in the databases using Geneious Pro version 5.0.4 software (Biomatters Ltd., Auckland, New Zealand). The synonymous substitution rate (*Ks*) and non-synonymous substitution rate (*Ka*), as well as the *Ka/Ks* ratios, were computed using DNAsp ver. 5.0 [35]. Divergence times were calculated by the mean divergence time, 2.7 million years ago (MYA) between the A and D genomes estimated by Dvorak and Akhunov [36]. Predicted proteins, as well as secondary structure predictions, were also obtained with this software, using the EMBOSS tool Garnier [37]. Amino acid substitutions between the predicted proteins obtained from the new alleles and reference proteins were analyzed using the PROVEAN (Protein Variation Effect Analyzer) software tool to predict whether these amino acid substitutions or InDels have an impact on their biological function [38,39].

A phylogenetic tree was constructed with the software MEGA6 [40] using the complete coding regions of the two sequences obtained, together with the following sequences of the *Wx* genes: common wheat CS (*Wx-A*1: AB019622, *Wx-B*1: AB019623, and *Wx-D*1: AB019624), two-rowed barley "Vogelsanger Gold" (*Wx-H*1: X07931) and six-rowed barley 'Morex' (*Wx-H*1: AF474373). A neighbor-joining cluster of all of the analyzed sequences was generated using the Poisson correction method for amino acid sequences [41] with one bootstrap consensus from 1,000 replicates [42].

3. Results

To determine the location of the *Wx* gene, different combinations of primers were used. However, for some of them, owing to similar fragment sizes, establishing the unambiguous presence of the *Wx* gene from *H. chilense* was difficult. The BDFL/BRD primers, designed by Nakamura et al. [33], provided the most reliable results (Figure 1).

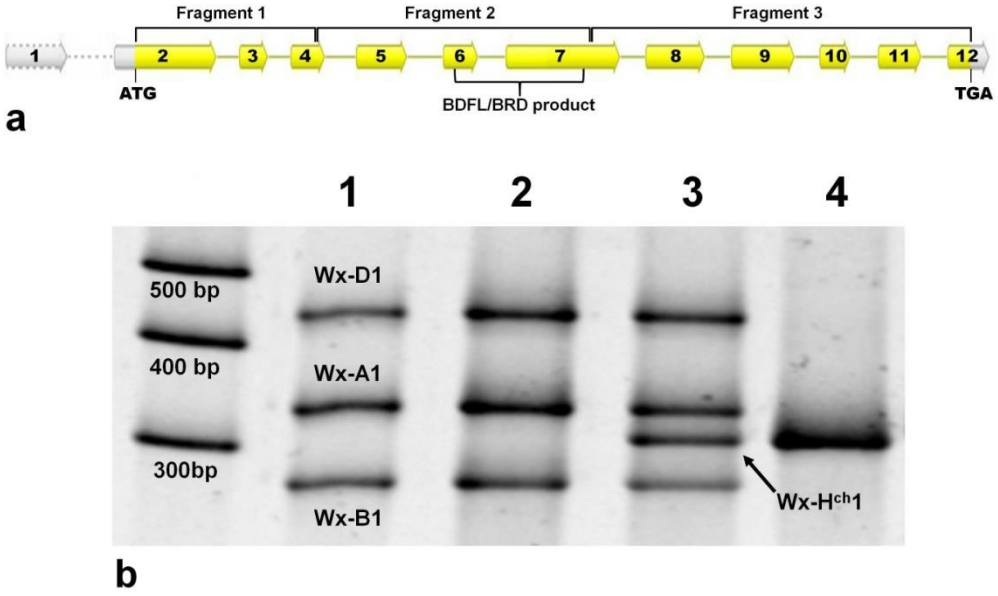

Figure 1. (**a**) Diagrammatic representation of *Wx* gene showing the three fragments used for sequencing, and (**b**) PCR analysis for the chromosomal location of *H. chilense Wx* gene using primers BDFL/BRD from Nakamura et al. [33] in common wheat, ditelosomic addition lines, and *H. chilense*. Lanes are as follows: 1, cv. Chinese Spring (CS), 2, CS + 7$H^{ch}S$ line, 3, CS + 7$H^{ch}L$ line, and 4, *H*1 line.

Because data obtained from other *Triticeae* species indicated that the *Wx* genes were mainly located on the short arm of the chromosome 7 [10], the CS + 7H^{ch}S and CS + 7H^{ch}L lines were used to determine the arm location of this gene in *H. chilense*. Figure 1 shows the presence of one additional band in the CS + 7$H^{ch}L$ line (Lane 3) that is a similar size to a band in the *H. chilense* (H1) line (Lane 4), which is absent in both the common wheat CS (Lane 1) and the CS + 7$H^{ch}S$ line (Lane 2). Thus, the *Wx-*H^{ch}1 gene is located on 7$H^{ch}L$ (H1), suggesting an inversion in this *H. chilense* chromosome.

The *Wx-*H^{ch}1 gene was analyzed in two lines of *H. chilense* that represent two different biotypes of this species present in Chile. Due to the length of this gene (~2700 bp), the genomic sequence was obtained amplifying three fragments, which covered the complete coding sequence. The first fragment of ~620 bp, covered part of the second exon (the first one in the coding sequence, see Figure 1) until the end of the fourth exon, while the second fragment (~960 bp) spanned the fourth to the seventh exons. Finally, the third fragment (~1160 bp) was the region between the end of fragment 2 and the 12th exon, including the TGA codon. The alignment and comparison are shown in Figure S1. The initiation codon, ATG, and the termination codon, TGA, for translation, as well as the splice junctions of each intron of *Wx-*H^{ch}*1*, were in homologous positions to those in other *Wx* genes. Both alleles detected in *H. chilense* (*Wx-*H^{ch}*1a* and *Wx-*H^{ch}*1b*) were smaller in size than the *Wx* genes used for comparison (Table 1).

The comparison between the seven nucleotide sequences showed that the greatest homology level was detected between the *Wx-*H^{ch}1 genes and the *Wx-H*1 variants from barley (90.8%). However, the comparison of the *Wx* genes from common wheat cv. Chinese Spring showed lower values of 83.6% for *Wx-D*1, 84.7% for *Wx-B*1 and 87.7% for *Wx-A*1. Nevertheless, the predicted proteins of these same sequences showed homology greater than 94% for all comparisons, and greater than 97.7% among barley species. This is in concordance with most of the sequence differences being found in introns. In all cases, the exons were the same size, with the exception of exon 2, which contained one or two additional codons in wheat but was similar in both *H. chilense* and *H. vulgare* (Table 1).

Table 1. Size of the different exons and introns of the coding sequence in the *Wx* sequences evaluated.

	Wx-A1a [1]	***Wx-B1a*** [1]	***Wx-D1a*** [1]	***Wx-H1a*** [2]/***Wx-H1b*** [3]	***Wx-H^{ch}1a/Wx-H^{ch}1b***
Exon 2	321	324	321	318	318
Exon 3	81	81	81	81	81
Exon 4	99	99	99	99	99
Exon 5	154	154	154	154	154
Exon 6	101	101	101	101	101
Exon 7	354	354	354	354	354
Exon 8	180	180	180	180	180
Exon 9	192	192	192	192	192
Exon 10	87	87	87	87	87
Exon 11	129	129	129	129	129
Exon 12	117	117	117	117	117
Intron 2	82	99	90	89	85
Intron 3	84	88	95	84	80
Intron 4	109	113	104	126	109
Intron 5	125	133	152	136	113
Intron 6	99	69	141	106	89
Intron 7	91	92	85	92	89
Intron 8	95	86	82	94	94
Intron 9	90	84	84	82	82
Intron 10	98	97	98	97	97
Intron 11	93	115	116	76	85/86
Total	2781	2794	2862	2794	2735/2736

[1] cv. Chinese Spring (NCBI ID: *Wx-A*1, AB019622, *Wx-B*1, AB019623, *Wx-D*1, AB019624) [43]. [2] cv. Vogelsanger Gold (NCBI ID: X07931) [12]. [3] cv. Morex (NCBI ID: AF474373).

3.1. Amino acid Predicted Sequence Analysis

While coding sequences of the *Wx* genes varied, most variation resulted in silent mutations that did not impact protein sequence or structure. Additionally, these proteins were synthesized as precursors or pre-proteins, including one transit-peptide of 70 amino acids and one mature domain. Nevertheless, potentially impactful sequence variation was detected in a conserved region of the mature domain related to waxy protein activity. These changes can lead to marked differences in the predicted sequences of the respective proteins (Figure S2).

The *Hordeum* sequences, including both *H. chilense* biotypes, showed the insertion of one amino acid residue within the signal peptide between positions 53 and 54. Furthermore, these sequences had deletions of Gly73 or Ala73 residues detected in the wheat proteins. Both InDels were the consequences of the aforementioned elimination of one or two codons in exon 2. Three non-conservative amino acid changes were detected in both *H. chilense* variants. For two of these changes, Pro24 → Arg and Ser416 → Pro, the H7 line showed the same amino acids as the other evaluated sequences. The Ser416 → Pro change could have deleterious effects according to the PROVEAN analysis, with a score of −2.869. The 419 position was different in both *H. chilense* sequences and was also different than the other sequences, with the exception of Wx-A1, which was similar to that of Wx-H^{ch}1a (Table 2).

The amino acid sequences from *H. chilense* were more similar to the waxy proteins from barley than those derived from any wheat genome. Only 15 changes were detected between *H. chilense* and barley variants, while up to 54 changes were observed when this comparison was carried out with wheat waxy proteins, and ten changes were common to both barley and wheat species (Figure S2).

Up to 15 amino acids variants were detected within the transit peptide. The sequence changes generated some variations in the secondary structure of the transit peptide. The most dramatic was the Val5 → Ala change (detected in barley waxy proteins but not in *H. chilense* ones) that resulted in an elongated first helix and the disappearance of a β strand in the secondary structure (Figure 2). With the exception of the aforementioned change in position 24 (Pro in Wx-H^{ch}1a and Arg in the others), all changes were common to both *H. chilense* variants. The barley variants showed three of

these conservative changes (Val5 → Ala, Ile18 → Val and Met68 → Val), although the waxy protein of cv. Vogelsanger Gold (*Wx-H1a* variant) included one additional non-conservative change at position 70 (Arg70 → Ser). Four of these differences, all classified as conservative, were common to Wx-B1 and Wx-D1 (Pro25 → Ala, Leu30 → Val, Asn34 → Ser, and Ser62 → Thr), although each variant showed two additional changes (Ala39 → Pro and Ile45 → Thr for Wx-B1, and Ile45 → Val and Lys52 → Thr for Wx-D1). Nevertheless, Wx-A1 was the most varied, with five unique changes (three conservative: Ile18 → Val, Ala58 → Pro and Gly61 → Phe, and two non-conservative: Gly17 → Ser and Ser62 → Asp) and one change in common with Wx-D1 (Ile45 → Val) (Figure 2).

Table 2. Amino acid comparison of predicted mature protein among waxy protein variants evaluated.

Position [1]	*Wx-H^{ch}1a/b*	*Wx-H1a* [2]	*Wx-A1a* [2]	*Wx-B1a* [2]	*Wx-D1a* [2]
103	Pro		Ala		
115	Val		Ile	Ile	Ile
123	Asn	Lys	Lys	Lys	Lys
131	Val	Ile	Ile		
137	Ala		Val		Val
139	Glu		Arg		Lys
142	Thr	Arg	Arg	Arg	Arg
145	Phe		Tyr	Tyr	Tyr
158	Ile		Val	Val	Val
162	Trp		Cys	Cys	Cys
189	Gln			Leu	
201	Ala		Val		Val
206	Asp	Asn			Asn
208	Asn				Asp
212	Tyr		His		
232	Pro	Leu	Leu	Leu	Leu
244	Asn			Ser	
249	Thr				Ala
356	Thr		Ile	Ala	
362	Ala		Thr		
363	Val			Ala	
367	Ile		Val	Val	
373	Ala		Gly	Gly	Gly
416	Ser/Pro	Pro	Pro	Pro	Pro
419	Val/Met	Leu		Leu	Leu
427	Ile		Val	Val	Val
438	Arg	Lys			
443	Val	Met			Ile
449	Gly		Thr	Ser	Ser
452	Arg		Trp		
471	Leu		Val	Val	Val
496	Ala	Val			
508	Val			Met	
535	Ala		Val	Val	Val
551	Gln		His	His	His
587	Ile			Val	Val
588	Val			Ile	Ile
590	Asp	Glu	Glu	Glu	Glu
597	Met		Leu		

[1] This position should be increased for *Wx-A*1 and -D1 (+1), and for *Wx-B*1 (+2). [2] NCBI ID: barley [X07931] and common wheat cv. "Chinese Spring" [*Wx-A*1: AB019622, *Wx-B*1: AB019623, *Wx-D*1: AB019624].

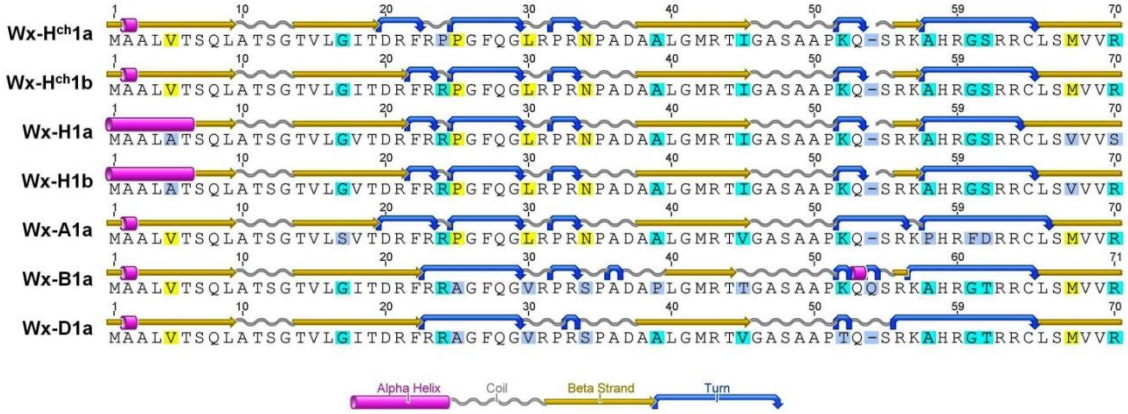

Figure 2. Comparison of the secondary structure motifs predicted by Garnier for the transit peptide region among all sequences evaluated.

Numerous changes were also observed in the mature proteins both in barley and wheat (Table 2). Five of these changes were common to both species (Asn123 → Lys, Thr142 → Arg, Pro 232 → Leu, Ser416 → Pro and Asp590 → Glu), whereas two changes were exclusively detected in barley (Arg438 → Lys and Ala496 → Val) and nine were exclusively detected in wheat (Val115 → Ile, Phe145 → Tyr, Ile158 → Val, Trp162 → Cys, Ala373 → Gly, Ile427 → Val, Leu471 → Val, Ala535 → Val and Gln551 → His). The other changes were detected in one or two wheat sequences (Table 2). Two of these changes could have effects on enzyme function (Figure 3). In addition to the abovementioned change, Ser416 → Pro, which was unique to Wx-H^{ch}1a, another change with deleterious effects predicted by the PROVEAN analysis was Pro232 → Leu, with a score of −4.061. The other changes observed were considered neutral.

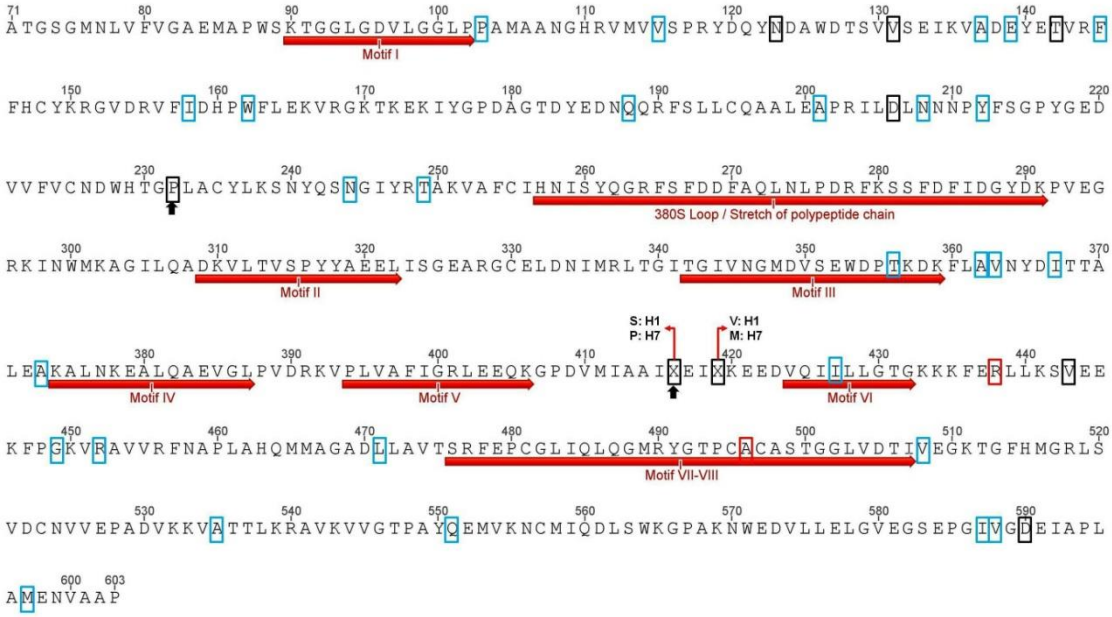

Figure 3. Consensus sequence of the predicted proteins from *H. chilense* showing the motifs described by Leterrier et al. [44] conserved in waxy proteins. Squares indicate substitution sites (blue for wheat, red for barley and black for both ones), and arrows point relevant changes found in the novel alleles.

Up to five of the eight motifs described by Leterrier et al. [44] are involved in the ADP glucose-binding and catalytic sites within the mature protein. Three changes were observed inside these conserved motifs (Figure 3). However, only the change Ile427 → Val was considered relevant because,

although this change was also detected in barley waxy protein, the wheat waxy proteins all showed Val as the residue in this position. The change Thr356 appeared in barley and the *Wx-D*1 protein, while Val 496 was exclusively found in the barley protein. The PROVEAN analysis suggested that these changes be considered neutral because their influence on the enzyme function was very limited.

3.2. Phylogenetic Analysis

The complete amino acid sequences of the two novel variants from *H. chilense* obtained in this study, together with other waxy protein sequences present in the NCBI database, were used to construct a phenogram based on the Poisson correction method for amino acid sequences (Figure 4). Three main clusters were observed, representing the correlations between the *H. chilense* sequences and the common barley sequences used.

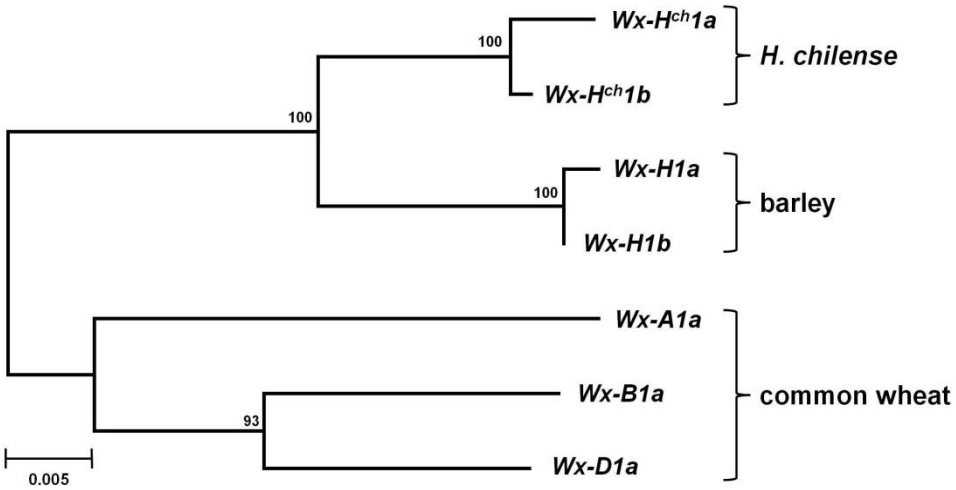

Figure 4. Neighbor-joining tree based on the Poisson correction method for amino acid sequences analyzed. Number above nodes indicates bootstrap estimates from 1000 replications.

These data were corroborated when the genomic nucleotide sequences of these variants were analyzed. Furthermore, the *Ks* and *Ka* substitution rates among *Wx* genes were calculated using the coding sequences of the complete genes. The comparison value between the genes from *H. chilense* and *H. vulgare* was high (*Ks* = 0.127), which suggested that the divergence time between species was ~2.3 MYA based on the mean divergence rate (0.0533 *Ks* per MY) obtained for this gene in a previous study [45].

4. Discussion

Knowledge regarding the influence of the amylose/amylopectin ratio on starch properties has encouraged the search for allelic variants that could increase/decrease either starch component. The most studied, in this context, has been the ADP glucose starch glycosyl transferase (GBSSI or the waxy proteins) solely responsible for amylose synthesis. This starch synthase has been studied in several cereal species, mainly those in which the starch properties are important for their use in the agri-food industry or in bio-ethanol production [46].

Recently, biotechnological techniques have allowed the development of new species using phylogenetically related species. These new species could also be used as a bridge to transfer new variations to common wheat. Thus, *H. chilense*, as a species involved in the synthesis of tritordeum, could be useful [25]. The incorporation of the H^{ch} genome in durum wheat has clear effects on the quality characteristics of the tritordeum. For example, the presence of the glutenins or hordeins of this wild species modifies the strength of the gluten in tritordeum flour [22], and their hordoindolines

change the texture of the grain from the ultra-hard of the parent durum wheat to soft in the derived tritordeum [29].

Here, we have studied one of the main keys in cereal flour quality, starch, by molecularly characterising the *Wx* gene in the two main lines of *H. chilense* used in the development of the tritordeums [25]. The *H. chilense* waxy proteins presented structures very similar to those of waxy proteins in other species of *Triticeae*, such as wheat and barley. The sizes of the predicted proteins were similar, although numerous amino acid changes were detected. However, these changes were mostly silent and not related to the active site of this enzyme, and probably, without influence on its function. In fact, some of these changes have been observed in other *Wx* genes [13]. The highly conserved structure of this gene makes it a good candidate for phylogenetic analysis [11,45,47–51]. In this study, the use of *Ka* established the separation between the *Hordeum* genomes at ~3 MYA.

In barley, Kramer and Blander [52] located the *Wx* gene on the short arm of chromosome 1 (7H). In common wheat, the waxy loci are located on chromosome 7AS (*Wx*-A1), chromosome 4AL, which was translocated from the original 7BS, (*Wx*-B1) and chromosome 7DS (*Wx*-D1) [10]. Here, the *Wx*-H^{ch}1 gene from *H. chilense* was located on $7H^{ch}L$, opposite the arm location found in the other Triticeae species [13]. Mattera et al. [53] indicated a similar change in the location of the *Phytoene syntase* (*Psy-1*) gene in *H. chilense*. *Psy*-1 was mapped in the distal region of $7H^{ch}S$, while this gene was located on the opposite arm of chromosome 7 in other Poaceae species [14]. On the basis of these changes, Mattera et al. [53] suggested that an inversion occurred between the distal parts of $7H^{ch}S$ and $7H^{ch}L$, which has been confirmed by Avila et al. [54]. The location of the *Wx*-H^{ch}1 gene on $7H^{ch}L$ in the present study supports this hypothesis on a structural change involving the distal regions of *H. chilense* chromosome $7H^{ch}$.

5. Conclusions

Variability in the *Wx* gene sequences was detected in two *H. chilense* lines representative of the two main ecotypes of species. This gene is located on the long arm of the $7H^{ch}$ chromosome, opposite to the other *Triticeae* species, which suggests the presence of an inversion between the distal parts of $7H^{ch}S$ and $7H^{ch}L$. Molecular characterization of these alleles showed that this gene is more similar to the *Wx* gene of barley than those of wheat. However, the enzymatic function would be similar in all species and, consequently, the variation present in *H. chilense* could be utilized in wheat breeding through the use of tritordeum as a bridge species.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/9/5/261/s1, Figure S1. Alignment of nucleotide sequences of the *Wx* alleles evaluated in this study, Figure S2. Alignment of predicted protein sequences of the waxy proteins evaluated in this study.

Author Contributions: J.B.A. conceived and designed the study; J.B.A., L.C., and R.R. performed the experiments; J.B.A. and A.C. analyzed the data and wrote the paper. All authors have read and approved the final manuscript.

Funding: This research was supported by grants AGL2014-52445-R and RTI2018-093367-B-I00 from the Spanish State Research Agency (Ministry of Science, Innovation and Universities), co-financed by the European Regional Development Fund (FEDER) from the European Union.

Conflicts of Interest: The authors declare no conflict of interest.

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