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ORIGINAL ARTICLE

# Molecular characterization of waxy alleles in three subspecies of hexaploid wheat and identification of two novel *Wx-B1* alleles

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## Abstract

***Key message*** **Two novel *Wx-B1 null* alleles that enlarge the genetic variability for this wheat gene were characterized, whose effects on wheat quality could be different to those of the *Wx-B1b* allele.**

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

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## Introduction

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

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

In recent decades, the role of different enzymes controlling starch composition/properties have been examined (Morell et al. 2001). Different starch synthases (SSI, SSII and SSIII) have been shown to function together with the branching (SBEI and SBEII) and debranching enzymes (SDBEs) in the synthesis of amylopectin, while the granule-bound starch synthase I (GBSSI) or waxy protein is the sole enzyme responsible for amylose synthesis. In common wheat (*Triticum aestivum* L. ssp. *aestivum*; $2n = 6 \times = 42$, BBAADD), one waxy protein for each genome has been detected—these Wx proteins are encoded by genes *Wx-A1*, *Wx-B1* and *Wx-D1* located on chromosomes 7AS, 4AL (translocated from original 7BS) and 7DS, respectively (Yamamori et al. 1994). Although different studies on the variation of these enzymes have been carried out and several variants of starch (amylose and amylopectin) synthesis enzymes have been detected (Yamamori et al. 1994, 1995; Yamamori and Endo 1996), variability in modern wheat cultivars is not very wide according to data in the Wheat Gene Catalogue (McIntosh et al. 2013). However, the use of the null variants detected has permitted the development of wheat lines with novel starch properties (Nakamura et al. 1995, 2006; Yamamori 2009; Yamamori and Quynh 2000; Yamamori and Yamamoto 2011), which have shown remarkable differences in terms of industrial (Graybosch 1998) and nutritional quality (Yamamori et al. 2006).

Species in the primary wheat gene pool could be good candidates as gene sources for the search of novel Wx variants to enable diversification of the starch properties of wheat. Within the hexaploid species of this gene pool, there are some neglected or underutilized subspecies, such as club, Indian dwarf or macha wheat, which have not been screened for waxy proteins variability. Club wheat [*T. aestivum* L. ssp. *compactum* (Host) Mackey], characterized by a compact spike, is distributed throughout the Old World (Filatenko and Hammer 2014) and has commercial importance in the US Pacific Northwest area for production of flours suitable for making cookies. The distribution areas of the other two subspecies are more limited. Indian dwarf wheat [*T. aestivum* L. ssp. *sphaerococcum* (Percival) Mackey] has small stature and small round grains and originated in India and Pakistan (Hosono 1954). Macha wheat [*T. aestivum* L. ssp. *macha* (Dekapr. & A.M. Menabde) Mackey] is a hulled wheat endemic to the Caucasus area. An important advantage of these three cultivated subspecies is that they cross readily with modern wheat and have little linkage drag of unwanted traits. Until now, several studies using Indian dwarf wheat have shown different interesting traits for genetic improvement, e.g. rust resistance (Chen et al. 2012), salt tolerance (Badridze et al. 2009), concentration of bioactive compounds in grain (Giambanelli et al. 2013) and even high grain yield (Zwer et al. 1995). Consequently, these wheat subspecies could be used to widen the genetic diversity of modern wheat for waxy proteins and other agronomic traits.

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

## Materials and methods

### Plant material

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

### Starch extraction and electrophoretic analysis

Whole grain flour from a single grain was mixed with 1 ml of distilled water and incubated at 4 °C for 24 h. The homogenate was filtered through Miracloth and centrifuged at 14,000*g* for 1.5 min. The pellet was washed with 1 ml of buffer A [55 mM Tris–HCl pH 6.8, 2.3 % (w/v) sodium dodecyl sulphate, 2 % (w/v) dithiotreitol, 10 % (v/v) glycerol], according to Echt and Schwartz (1981). Then 1 ml of buffer A was added to the pellet and left for 30 min at room temperature. The pellet was washed three times with distilled water, once with acetone and then air-dried. The residue was mixed with 80 µl of buffer A containing 0.02 % (w/v) bromophenol blue, heated in a boiling bath for 2 min, cooled in ice and centrifuged.

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

For two-dimensional polyacrylamide-gel electrophoresis (2D-PAGE), starch purified as described above was incubated at room temperature in 300 µl of lysis buffer [8 M urea, 2 % Triton X-100, 2 % ampholine pH 3.5–10 (Pharmacia LKB) and 5 % 2-mercaptoethanol]. After centrifugation, the supernatant containing the solubilised proteins was subjected to 2D-PAGE using isoelectric focusing (IEF) for the first dimension and modified SDS-PAGE for the second (Nakamura et al. 1993). IEF gels contained 2.5 % (v/v) ampholines (pH 3.5–10/5–8, 1:1). Focusing was begun from the acidic end (0.01 M $H_3PO_4$) and continued at 400 V for

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

| Primers designed by Guzmán and Alvarez (2012) | |
|---|---|
| Wx1 | Fw: 5′-TTGCTGCAGGTAGCCACACC-3′<br>Rv: 5′-CCGCGCTTGTAGCAGTGGAA-3′ |
| Wx2 | Fw: 5′-ATGGTCATCTCCCCGCGCTA-3′<br>Rv: 5′-GTTGACGGCGAGGAACTTGT-3′ |
| Wx3 | Fw: 5′-GGCATCGTCAACGGCATGGA-3′<br>Rv: 5′-TTCTCTCTTCAGGGAGCGGC-3′ |
| Primers designed by Nakamura et al. (2002) | |
| BDFL | 5′-CTGGCCTGCTACCTCAAGAGCAACT-3′ |
| BRD | 5′-CTGACGTCCATGCCGTTGACGA-3′ |
| Primer designed in this study | |
| Wx1.3Rv | 5′-TAGCGCGGGGAGATGACCAT-3′ |

PCR conditions

Initial denaturation = 3 min at 94 °C

| | Pair | Denaturation | Annealing | Extension |
|---|---|---|---|---|
| 35 cycles | Wx1 [Fw/Rv] | 40 s at 94 °C | 30 s at 64 °C | 1 min at 72 °C |
| | Wx2 [Fw/Rv] | 30 s at 94 °C | 30 s at 66 °C | 1 min 30 s at 72 °C |
| | Wx3 [Fw/Rv] | 40 s at 94 °C | 30 s at 64 °C | 1 min 30 s at 72 °C |
| | BDFL/BRD | 30 s at 94 °C | 30 s at 65 °C | 2 min at 72 °C |
| | Wx1Fw/Wx1.3Rv | 30 s at 94 °C | 20 s at 66 °C | 20 s at 72 °C |

Final extension = 5 min at 72 °C

15 h and then 800 V for 60 min at room temperature. Proteins were revealed by silver staining according to Silver stain kit (Wako Pure Chemical Industries, Ltd., Japan).

### DNA extraction and PCR amplification

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

The genomic sequence of the *Wx* gene contains twelve exons and eleven introns, with a coding region around 2800 bp. Primers designed by Guzmán and Alvarez (2012) were used to amplify the coding region of *Wx* genes in three regions or fragments: the first from second to fourth exon (Wx1Fw/1Rv); the second from fourth to the seventh exon (Wx2Fw/2Rv); and the last fragment from the seventh to the twelfth exon (Wx3Fw/3Rv).

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

### Analysis of PCR products, cloning and sequencing analysis

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

## Results

### Waxy protein polymorphism and PCR analysis

The SDS-PAGE electrophoresis of waxy proteins did not detect polymorphism for Wx-A1 or Wx-D1 proteins in any

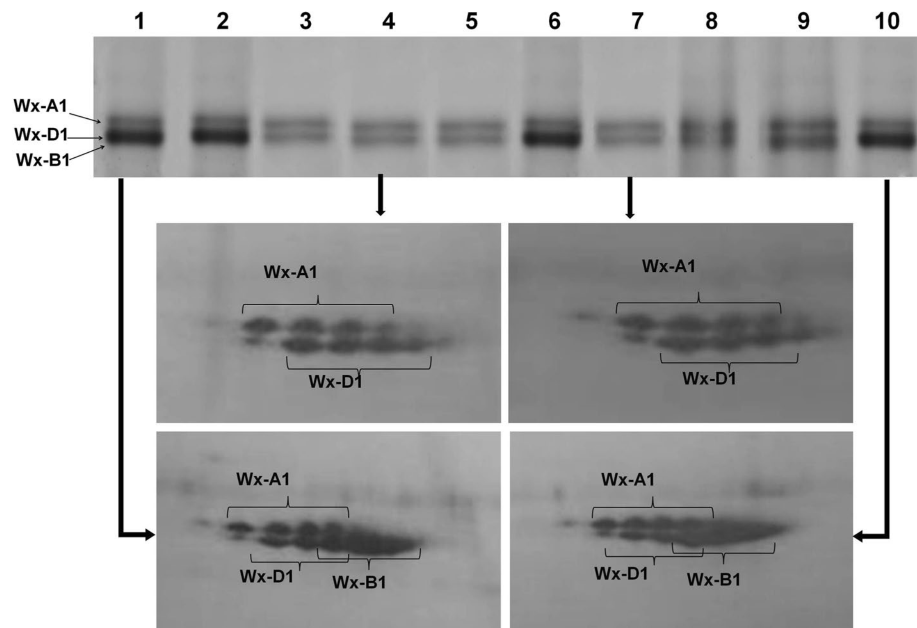


**Fig. 1** Waxy protein polymorphism by SDS-PAGE and 2D electrophoresis means. All the samples had Wx-A1 and Wx-D1 proteins. *Lanes* are as follows: *1* PI 355512, *2* PI 422411, *3* PI 357307 (lack of Wx-B1), *4* PI 442911 (lack of Wx-B1), *5* PI 565431 (lack of Wx-B1), *6* cv. Chinese Spring, *7* PI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

of the three species. All samples showed two bands with the same mobility as those of cv. Chinese Spring (Fig. 1a). However, five accessions of club wheat and 13 of Indian dwarf wheat lacked the Wx-B1 protein (ESM-1). In contrast, all accessions of macha wheat showed a band similar to Wx-B1 protein of Chinese Spring. These results were confirmed by 2D-PAGE (Fig. 1b).

To identify the cause of the lack of Wx-B1 protein, PCR markers described by Nakamura et al. (2002) that permit identification of the *Wx-B1b* allele (the most common *Wx-B1* null allele) were used to screen these accessions. In general, accessions that produced the three waxy proteins had the three PCR product bands corresponding to each *Wx-1* gene (Fig. 2, lane 4). Compositions of the 18 accessions that did not show the wild composition are presented in Table 2. Thirteen of the accessions lacking Wx-B1 protein did not show the corresponding *Wx-B1* band as did cv. Kanto 107 (Fig. 2, lane 8), a positive control for the *Wx-B1b* allele (Table 2). However, five of the accessions showed a PCR product corresponding to *Wx-B1*. The accession PI 442911 showed a band of the same apparent size as for Chinese Spring (Fig. 2, lane 3), and accessions CItr 4923, PI 272580, PI 330556 and PI 352499 showed a *Wx-B1* band with less electrophoretic mobility (Fig. 2, lane 5).

### Wx-B1 sequences analysis

The *Wx-B1* genes of accessions that showed a PCR product different from the *Wx-B1b* allele were cloned and

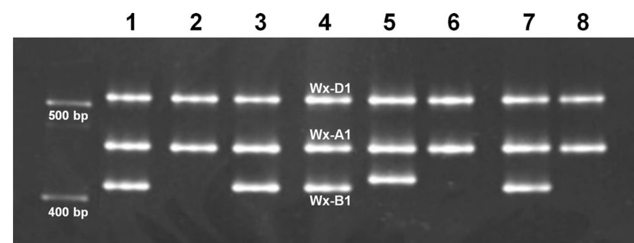


**Fig. 2** PCR analysis using primers BDFL/BRD from Nakamura et al. (2002). *Lanes* are as follows: *1* PI 422411, *2* PI 357307 (lack of Wx-B1), *3* PI 442911 (lack of Wx-B1), *4* cv. Chinese Spring, *5* PI 272580 (lack of Wx-B1), *6* PI 272581 (lack of Wx-B1), *7* PI 278650, and *8* Kanto 107

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

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

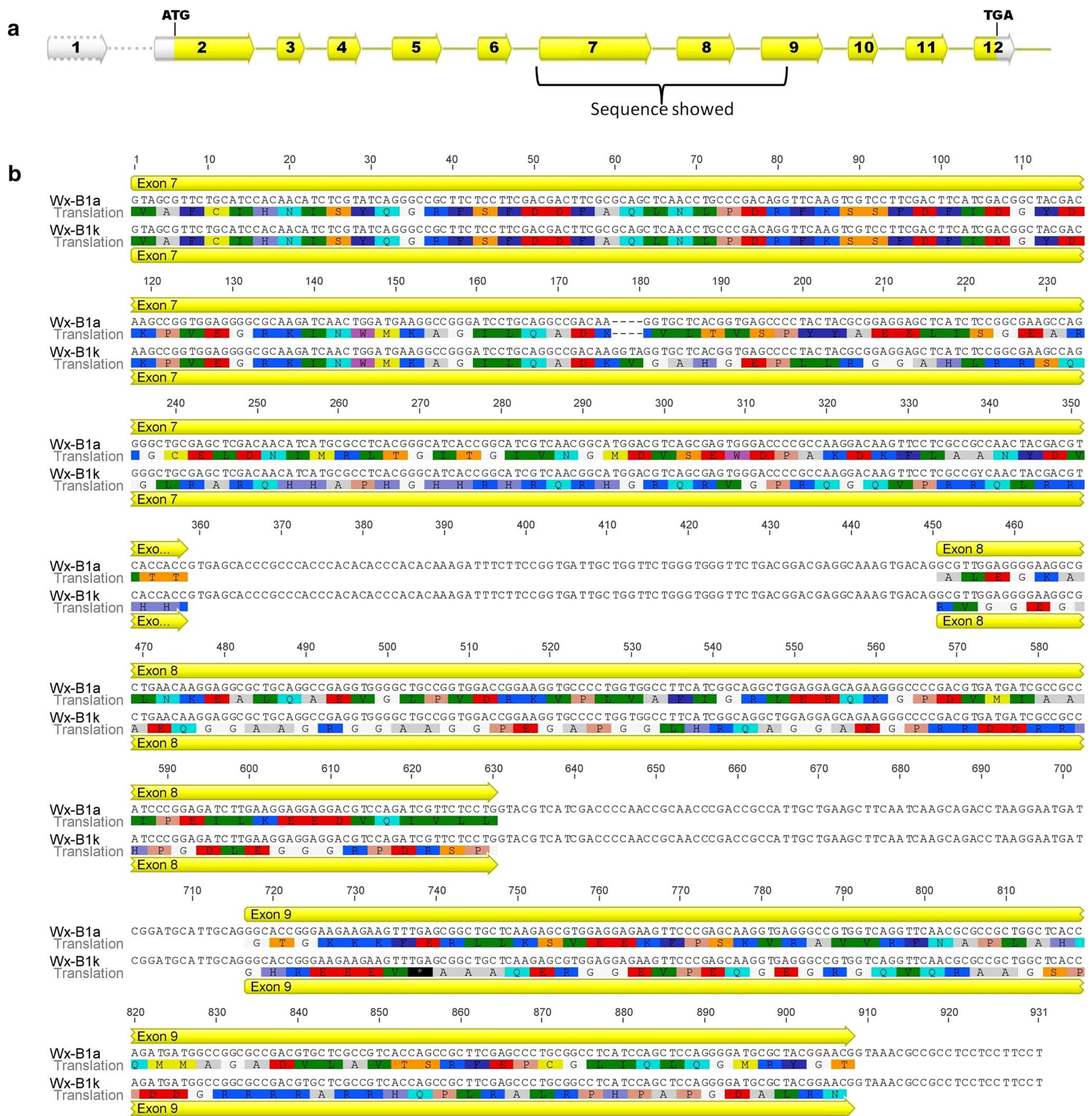


**Fig. 3** Molecular characterization of the novel *Wx-B1k null* allele. **a** Diagrammatic representation of the *Wx-B1* gene. *Yellow regions* encode waxy protein. *Dotted regions* are not analyzed. **b** Comparison of genomic DNA sequences and deduced amino acid sequences of *Wx-B1a* and *Wx-B1k*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

sequenced to identify the reason for the absence of the Wx-B1 protein. The sequences obtained were compared to the *Wx-B1a* allele of Chinese Spring. The four accessions of Indian dwarf wheat showed the same mutation, carrying the insertion of four nucleotides (GGTA) in the seventh exon at position 1437 from the start codon (Fig. 3). This insertion caused a frameshift mutation that generated a stop codon (TGA) within the ninth exon, which would make a truncated protein. Comparing this sequence with others available in NCBI GenBank by BLAST revealed that this mutation was novel and, therefore, the allele was provisionally named *Wx-B1k* (NCBI ID: KP726909), according to international nomenclature (McIntosh et al. 2013).

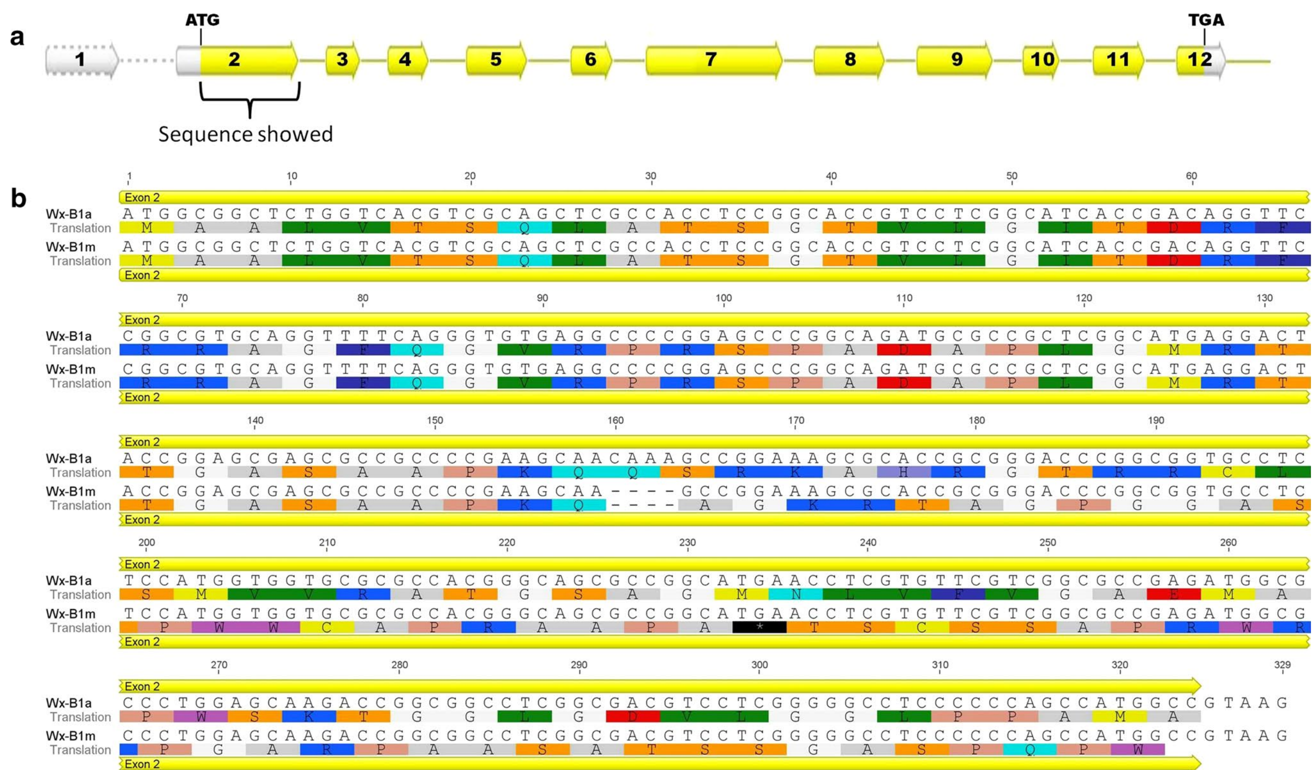


**Fig. 4** Molecular characterization of the novel *Wx-B1m null* allele. **a** Diagrammatic representation of the *Wx-B1* gene. *Yellow regions* encode waxy protein. *Dotted regions* are not analyzed. **b** Comparison of genomic DNA sequences and deduced amino acid sequences of *Wx-B1a* and *Wx-B1m*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

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

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

Because the variation of the novel *Wx-B1m* allele was detected in the beginning of the gene, the sequence between positions −31 and 466 was amplified by the use of the Wx-1Fw primer together with a new reverse primer (Wx1.3Rv, Table 1). Three products of 492 bp (*Wx-B1a*), 480 bp (*Wx-D1a*) and 472 bp (*Wx-A1a*) were expected to be obtained in Chinese Spring by PCR with this primer pair. The PCR product was run in a polyacrylamide gel (Fig. 5, lanes 1-3). Chinese Spring (lane 1) showed only two bands, indicating that probably two of the *Wx-1* products (*Wx-A1* and *Wx-D1*) were co-migrating. This is in consonance with the profile of Kanto 107 (lane 3), which lacked the whole of *Wx-B1* gene, and its *Wx-A1b* gene is 23 nucleotides smaller than the *Wx-A1a* allele of Chinese Spring (Vrinten et al. 1999). In any case, the

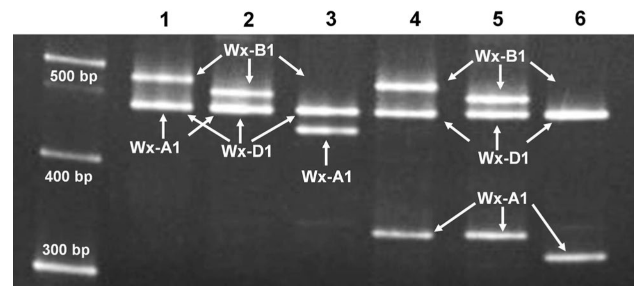


**Fig. 5** Molecular marker to detect *Wx-B1m* allele. In *lanes 1–3* PCR products resulted from the amplification with primers Wx1-Fw and Wx1.3Rv. In *lanes 3–6* the amplification products are digested with endonuclease *Dde*I, which has target sequences in *Wx-A1* amplicon. *Lanes* are as follows: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

co-migration of both bands was confirmed by digesting the PCR product with the *Dde*I endonuclease, which had target sequences only in the *Wx-A1* product (lanes 4–6). The PCR digestion confirmed that the lower band of Chinese Spring (lane 1) and PI 442911 (lane 2) was composed by *Wx-A1* and *Wx-D1* products. Therefore, the upper band corresponded to *Wx-B1*, which presented two variants in addition to those of Kanto 107. In genotype PI 442911 (*Wx-B1m*), the *Wx-B1*

band showed higher mobility than those of Chinese Spring (*Wx-B1a*), due to the deletion of four nucleotides as described above. Consequently, the use of this PCR assay permitted discrimination among *Wx-B1a*, *-B1b* and *-B1m* alleles.

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

## Discussion

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

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

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

A contrasting discrepancy was observed in the analyzed accessions of Indian dwarf wheat. In this study, some accessions (PI 282451, PI 282452, PI 324492 and PI 352498) were found to carry the *Wx-B1b* null allele according to protein electrophoresis as well as PCR using the BDFL and BRD primers. These results were in agreement with those of Li et al. (2013); however, they described PI 272580, PI 330556 and PI 352499 as having the wild allele for *Wx-B1*. We demonstrated here that these accessions had a novel *Wx-B1k null* allele by molecular characterization of the *Wx-B1* gene. The molecular characterization was carried out because the protein electrophoresis analysis (SDS-PAGE and 2D) revealed that these accessions lacked the Wx-B1 protein and this was not in agreement with the result obtained with the BDFL and BRD primers. This fact strengthens the idea that for appropriate evaluation of waxy protein variability, both protein and DNA analysis (PCR and/or sequencing) should be combined for waxy protein/gene alleles to avoid misclassification due to failures or inconclusive results with only one method (Ortega et al. 2015).

In our study, the analysis of a set of club wheat showed five accessions lacking Wx-B1 protein, four of which had the *Wx-B1b null* allele as detected with BDFL and BRD primers. The other accession had a novel allele (*Wx-B1m*), characterized by the deletion of four nucleotides in the second exon. As for *Wx-B1k*, this produced a frameshift in the ORF (i.e. premature appearance of a stop codon) which would result in absence of the protein. This kind of frameshift mutation leading to lack of the protein has been described several times in *Wx-A1* (Saito et al. 2004; Saito and Nakamura 2005; Vanzetti et al. 2010), but only once

in *Wx-B1* (Guzmán et al. 2015). This is important because the common *null* mutation for *Wx-B1* (*Wx-B1b*) implies the deletion of the entire gene and the surrounding region (67 kb), in which other genes related to quality could be included (Saito et al. 2009). Additionally, Wx-B1 protein has been shown to have a greater impact on amylose synthesis compared to Wx-A1 and Wx-D1 (Miura and Sugawara 1996; Yamamori and Quynh 2000), so the detection of variability is important to provide more sources of variation for starch modification.

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

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

**Author contribution statement** JBA and CG conceived and designed the study. MA performed the experiments. MY performed the 2-D PAGE analysis. All authors analyzed the data and wrote the paper. All them have read and approved the final manuscript.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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