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# Puroindoline (*Pina-D1* and *Pinb-D1*) and waxy (*Wx-1*) genes in Iranian bread wheat (*Triticum aestivum* L.) landraces

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**ABSTRACT**

Grain hardness and starch are two of the most important factors that determine the end-use quality of bread wheat (*Triticum aestivum* L.) grain. The grain hardness and amylose content are controlled by the puroindolines (*Pina-D1* and *Pinb-D1*) genes, located on chromosomes 5D, and waxy (*Wx-A1*, *-B1* and *-D1*) genes, located on chromosomes 7A, 4A and 7D. A total of 160 Iranian landraces from the Germplasm Bank of the International Maize and Wheat Improvement Center were evaluated for grain hardness using near infrared spectroscopy to predict the particle size index (PSI). In addition, molecular markers were used to evaluate the states of the corresponding genes. Eight accessions were found to have a hard texture (predicted PSI $<$ 45%); however, only two could be explained by null alleles either in *Pina-D1* or *Pinb-D1*. Additionally, 152 accessions had semi-hard textures (predicted PSI range of 45% to 55%). For the *Wx* gene, only one accession (CWI 67665) showed the null *Wx-D1b* allele, while two accessions (CWI 67747 and CWI 57684) were null for *Wx-B1b*. Single nucleotide polymorphism and sequence tag site marker techniques were used. Our findings further indicate the importance of using these landraces for grain quality improvement in breeding programs.

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Puroindoline; Waxy proteins; landraces; KASP markers; Triticum aestivum

## Introduction

Bread wheat (*Triticum aestivum L. ssp. aestivum*; 2n = 6x = 42, AABBDD) is an important crop worldwide and is estimated to be the staple food for 2.5 billion people in 89 countries (more than 30% of the world population) [1]. Grain hardness, starch properties and dough (gluten) viscoelastic properties are quality aspects that explain most of the variation in wheat grain quality traits [2–4]. Grain hardness or texture is one of the most important single factors determining the end-use food properties of wheat grain [5–7], and it also determines the marketing of wheat grain [8]. On the basis of this trait, wheat is classified into three main classes: very hard (durum wheat), hard and soft (bread wheat) [9]. Hard and soft wheat mill differently. Compared with soft wheat flours, hard wheat flours have greater levels of damaged starch, which leads to increased water absorption, something desirable for bread making [10, 11]. In general, hard wheat is used for making bread and soft wheat is used in the manufacture of cookies, cakes and pastries [7, 8].

Grain hardness is genetically controlled by genes which have been reported to be located at the hardness locus (*Ha*) in the distal end of the short arm of chromosome 5D in bread wheat [8, 12–14], but in durum wheat (*Triticum turgidum ssp. durum* Desf. em. Husn.; 2n = 4x = 28, AABB) and other tetraploid species, these genes are not present, because of the absence of the D genome, and the complete deletion of these genes in the A and B genomes during the evolution of tetraploid wheat [13]. Studies have shown that the hardness (*Ha*) locus of bread wheat has a complex structure within an 82-kb region [13]. This includes two genes (*Pina-D1* and *Pinb-D1*) that code for two basic grain proteins, the puroindoline a and b (Pina and Pinb) of ~13 KDa, together with the grain softness protein. The Pins (a and b) are unique among

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other plant proteins because of their basic cysteine-rich nature and the tryptophan-rich domains [6]. The presence of wild type alleles (*Pina-D1a* and *Pinb-D1a*) is correlated with soft grain in bread wheat, whereas nucleotide changes in the coding regions or deletions of whole *Pin-D1* genes (null alleles) are correlated with a hard texture [15–18]. The gene polymorphisms lead to differences in the degree of hardness [19]. The literature indicates that genotypes harboring *Pina-D1b*/*Pinb-D1a* have harder grains than genotypes that carry *Pina-D1a*/*Pinb-D1b* [20–24].

Starch composition is another parameter affecting the processing and end-use quality of wheat grain. This macromolecule is composed of two types of glucose polymers, amylose and amylopectin [25], with a ratio of 20%-30% amylose to 70%-80% amylopectin [26]. The physical and chemical properties of starch (gelatinization, pasting and gelation) depend on the relative amounts of amylose and amylopectin [27, 28]. Therefore, starches with different amylose/amylopectin ratios are needed for various industrial applications [29, 30]. Environmental factors (such as temperature) and growth conditions can influence this ratio [31]. Granule-bound starch synthase I or waxy (*Wx*) protein is the key enzyme in amylose synthesis and is encoded in bread wheat by the *Wx-A1*, *-B1* and *-D1* genes, which are located on the 7A, 4A and 7D chromosomes [32–34].

In recent years, several studies have been conducted to identify new waxy alleles in different wheat-related species, such as einkorn and Aegilops [35, 36] and in landraces [37] to increase the genetic resources used to develop wheat varieties with novel starch properties and end-use quality characteristics. Wheat landraces are important sources of genetic diversity that can improve the gene pools of modern cultivars by introducing new alleles [38–42]. Iran is one of the primary habitats of wheat's ancestors and is, therefore, a reservoir for new alleles. The International Maize and Wheat Improvement Center (CIMMYT) gene bank possesses ~150,000 wheat and related species accessions, among which 48,600 accessions are of traditional durum and bread wheat landraces. This includes 6,947 accessions from Iran. The main aim of this study was to determine the distribution of *Pin-D1* and *Wx-1* alleles in a collection of Iranian bread-wheat landraces from the CIMMYT Germplasm Bank.

## Materials and methods

### Plant materials

A total of 160 Iranian bread-wheat landraces, held as accessions in the germplasm bank of the CIMMYT (Texcoco, Edo. de Mexico, Mexico) (Supplemental Table S1) were used in the study. The accessions were evaluated at the experimental field station 'CENEB' located near Ciudad Obregon, Sonora, Northwest Mexico (27_209 N, 109_549 W, 38 m above sea level), during the 2010 – 2011 cropping cycle under optimum growing conditions (full irrigation).

### Quality parameters

Quality parameters were determined using the methods established by the American Association of Cereal Chemists [43]. The grain protein content (GPC, 12.5% moisture basis) and hardness particle size index (PSI, %) were determined using a near infrared (NIR) spectroscopic NIR System 6500 (FOSS-Tecator, HillerØd, Denmark), calibrated for protein content using the Kjeldahl method [43] and for hardness using the PSI method [44]. Soft wheat endosperm produces a greater proportion of fine particles that correspond to higher PSI percentages. Based on the predicted PSI data obtained by NIR, samples were classified as hard (30%-44%), semi-hard (45%-55%) and soft (>55%).

### Genomic DNA extraction

Genomic DNA was extracted from young leaves of two-week-old seedlings grown in a greenhouse using a modified CTAB method described in Dreisigacker *et al.* [45]. The quality and quantity of DNA were evaluated in 1% agarose gels and using a Nanodrop 8000 spectrophotometer (Thermo Scientific, USA) and the samples were diluted to a final concentration of 50 ng/mL.

### Sequence tag site (STS) markers

Sequences of PCR primers and fragment sizes are shown in Table 1. Each 10-μL reaction included 50 ng DNA, 1.5 mmol/L MgCl2, 0.6 μmol/L of each primer, 0.8 μmol/L dNTPs, 1.5 ml 2.0× PCR buffer and 0.05 U Taq polymerase (Go Taq Flexi, Promega Crop., Cat. #M8295). PCR was performed in an ABI Genamp 9700 PCR Thermocycler (Applied Biosystems, USA). The PCR conditions included an initial denaturation step of 2 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 2 min at 60 °C and 2 min at 72 °C. There was then a final 5-min extension at 72 °C. The amplification products were separated in 2.5% agarose gels.

**Table 1.** List of STS markers used in this study.

| Trait | Locus/Gene | Allele | Marker | Size (bp) | Chr. | Source |
|---|---|---|---|---|---|---|
| Grain hardness | *Pina-D1* | a/b | pinaD1 | 349 | 5D | Gautier et al.1994 [6] |
| | *Pinb-D1* | a(wild type) | PB5/SR | 250 | 5D | Gautier et al.1994 [6] |
| | *Pinb-D1* | Null | PB5/HR | 250 | 5D | Gautier et al.1994 [6] |
| Starch properties | *Wx-A1* | a/b | MAG264 | 336a/317b | 7A | Liu et al.2005 [46] |
| Starch properties | *Wx-D1a/b* | a/b | MAG269 | 1400/800 | 7D | Liu et al.2005 [46] |
| Starch properties | *Wx-B1* | a/b | GBSS | 320 | 4A | Mclauchlan et al.2001 [47] |
| Starch properties | *Wx-B1* | a(wild type) | BDFL + BRC1 | 778 | 4A | Saito et al.2009 [48] |
| Starch properties | *Wx-B1* | b(null type) | BFC + BRC2 | 668 | 4A | Saito et al.2009 [48] |

**Table 2.** Kompetitive allele PCR assays for used in this study.

| | | | | *FAM-allele* | | | *VIC-allele* | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Trait* | *Gene* | *Assay* | *Polymorphism* | Call | Allele | Phenotype | Call | Allele | Phenotype | Reference |
| *Grain hardness* | *Pina-D1* | *Pina-D1_INS* | A/G | A | *Pina-D1a* | Soft | G | *Pina-D1b* | Hard | Giroux and Morris (1998) [50] |
| | *Pinb-D1* | *Pinb-D1-INS* | C/T | C | *Pinb-D1a* | Soft | T | *Pinb-D1b* | Hard | Giroux and Morris (1997) [15] |
| *Starch properties* | *Wx-B1* | *Wx-B1_INS* | InDel | Del | *Wx-B1a* | Waxy-type | Ins | *Wx-B1b* | Null | Saito et al. (2009) [48] |

**Table 3.** Allelic compositions of puroindoline genes and the PSI values of the Iranian bread-wheat landrace accessions.

| Phenotype | Puroindoline genes | *N* | PSI (%+s.d[a]) | Range (%) |
|---|---|---|---|---|
| Hard (30%–44%) | *Pina-D1a/Pinb-D1a* | 6 | 42.3 ± 1.4 | 41–43 |
| | *Pina-D1null/Pinb-D1a* | 1 | 43 | |
| | *Pina-D1a/Pinb-D1null* | 1 | 41 | |
| Semi-Hard (45%–55%) | *Pina-D1a/Pinb-D1a* | 147 | 50.4 ± 2.42 | 46–54 |
| | *Pina-D1b/Pinb-D1a* | 1 | 53 | |
| | *Pina-D1a/Pinb-D1null* | 4 | 49.5 ± 2.38 | 47–52 |

[a]Standard deviation.

### *Kompetitive allele-specific PCR (KASP) markers*

KASP is single-step genotyping technology that detects, using a fluorescence-based application, pre-identified co-dominant and dominant alleles of both single nucleotide polymorphism (SNP) and insertion/deletion variants [49]. The primer sequences and more detailed allelic information are provided in Table 2. For *Pina-D1* and *Pinb-D1*, KASP assays were conducted in a 384-well format and performed in 4.2 μL reactions containing 2.1 mL sterile water, 2 mL 2× KASP Mix, 0.1 μL assay mix and 50 ng of dried DNA. For *Wx-B1*, KASP assays were performed using optimized buffer that contained $MgCl_2$ (50 nm). PCR amplification was performed in a GenAmp PCR system 9700 Thermal cycler (Applied Biosystems) using the following cycling conditions: 94 °C for 15 min for hot-start Taq DNA polymerase activation, 11 cycles at 94 °C for 30 s, 65 °C for 60 s (-0.8 °C each cycle) and 72 °C for 30 s, followed by 26 cycles at 94 °C for 30 s, 57 °C for 60 s and 72 °C for 30 s. Then, there was a final extension at 72 °C for 5 min. End-point fluorescent images were visualized using PHERAstar$^{plus}$ (BMG LABTECH, Germany), and the data were analyzed using KLuster Caller$^{TM}$ software (LGC Genomics).

## Results and discussion

### *Grain hardness*

A wide range of predicted PSI values was identified for the accessions (Table 3), with most of the samples (152) having semi-hard textures (predicted PSIs ranging between 45% and 55%). Eight accessions had hard textures (predicted PSIs < 45%) and none had a soft texture (PSI > 55%).

### *STS marker analysis*

Primers from Gautier *et al.* [6] were used to amplify *Pina-D1* and *Pinb-D1* genes and detect possible differences in amplicon presence/absence and size. The amplification of Pina-D1 yielded an expected PCR product of ~349 bp. Most of the accessions produced a PCR product of ~349 bp (*Pina-D1a*), except for two accessions (CWI56592 and CWI72061), which produced no amplification products, indicating that they were *Pina-D1b(a*-null) (Figure 1a). These two accessions were classified as hard and semi-hard based on the predicted PSI values. For the *Pinb-D1* gene, most of the accessions produced a PCR product of 250 bp (*Pinb-D1a*) (Figure 1b), except for five accessions (CWI

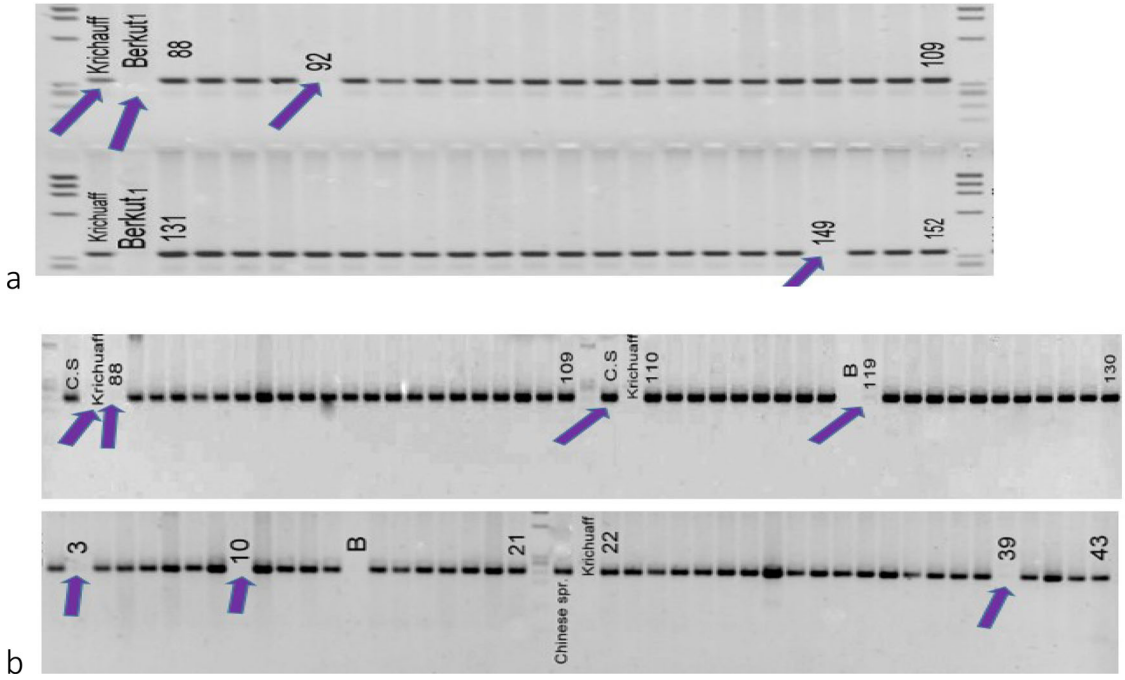


**Figure 1.** PCR analysis of Iranian bread-wheat landraces using STS primers from Gautier *et al.* [6].
(**a**) *Pina-D1* amplification in landraces Krichauff (*Pina-D1a*/*Pinb-D1*null), Berkut1 (*Pina-D1b*/*Pinb-D1a*), number 92 CWI720619 (*Pina-D1b*/*Pinb-D1a*) and number 149 CWI56592 (*Pina-D1b*/*Pinb-D1a*). (**b**) *Pinb-D1* amplification in landraces Chinese Spring (*Pina-D1a*/*Pinb-D1a*), Krichuff (*Pina-D1a*/*Pinb* null), number 3 CWI 73113 (*Pina-D1a*/*Pinb-D1* null), number 10 CWI 67068 (*Pina-D1a*/*Pinb-D1* null), number 39 CWI 72525 (*Pina-D1a*/*Pinb-D1* null), number 88 CWI 71600 (*Pina-D1a*/*Pinb-D1* null) and number 119 CWI 57139 (*Pina-D1a*/*Pinb-D1* null).

73113, CWI 67068, CWI 72525, CWI 71600 and CWI 57139). The lack of product was considered as an indication of the absence of *Pinb-D1* (*Pinb* null). Of these accessions, CWI 73113 was classified as hard and CWI 67068, CWI 72525, CWI 71600 and CWI 57139 were classified as semi-hard based on the predicted PSI values.

For the *Wx* loci, all the accessions contained the alleles *Wx-A1a* and *Wx-D1a* in experiments using the markers MAG264 and MAG269, respectively, except for one accession (CWI67665) that had no amplification product, indicating *Wx-D1b* (null mutation) (Figure 2a). At the *Wx-B1* locus, tested using marker *Wx-B1*, all the accessions contained the allele *Wx-B1a*, except for two accessions (CWI67747 and CWI57684), indicating *Wx-B1b* (null mutation) (Figure 2b and c).

## KASP marker analysis

KASP primers were used in this study to identify the *pina-D1*/*pinb-D1* and *Wx-B1* genes based on sequences upstream and downstream of the detected SNPs (with the 3′ end of the forward primer positioned at the mutant SNP site). For *Pina-D1*, two accessions (CWI 56592 and CWI 72061) shown in red (VIC) with null type alleles pina-D1b (A/G SNP) produced a hard texture in bread wheat. For *Pinb-D1*, five accessions (CWI 73113, CWI 57816, CWI 72525, CWI 71600 and CWI 57139) shown in red (VIC) with Pinb-D1b alleles (C/T SNP) produced hard textures. This identification is due to the codon change Gly-46 to Ser-46 and other accessions shown in blue (FAM) with wild-type alleles (*pina-D1a* and *pinb-D1a*) produced soft textures in bread wheat. No heterozygosity was detected (Figure 3b). For *Wx-B1a*, only one accession (CWI 67747) shown in red (VIC) had the null allele (*Wx-B1b*), and all the accessions shown in blue (FAM) had the wild-type allele (*Wx-B1a*) (Figure 3c).

Bread-making quality is a key target of breeding programs. It is controlled by wheat genetics and the environment. Grain hardness influences wheat milling and flour viscoelastic properties [51–54], and it is used for marketing classifications [8]. In the present study, a collection of Iranian bread-wheat landraces held in the CIMMYT's wheat germplasm bank was analyzed for grain hardness using NIR calibrated by PSI and characterized at the molecular level by Pin compositions and *Wx* genes. The accessions were classified into hard and semi-hard wheat. In eight of the accessions showing a hard grain texture, two could be explained by a null mutation in either *Pina-D1* or *Pinb-D1*, which corroborated earlier findings [24, 55, 56]. Accessions harboring *Pina-D1* null alleles may be harder than those with other Pin alleles, such as Pinb-D1b [23, 57], and have low milling yields [19, 22, 23]. This mutation has been reported in numerous studies worldwide [16, 24,

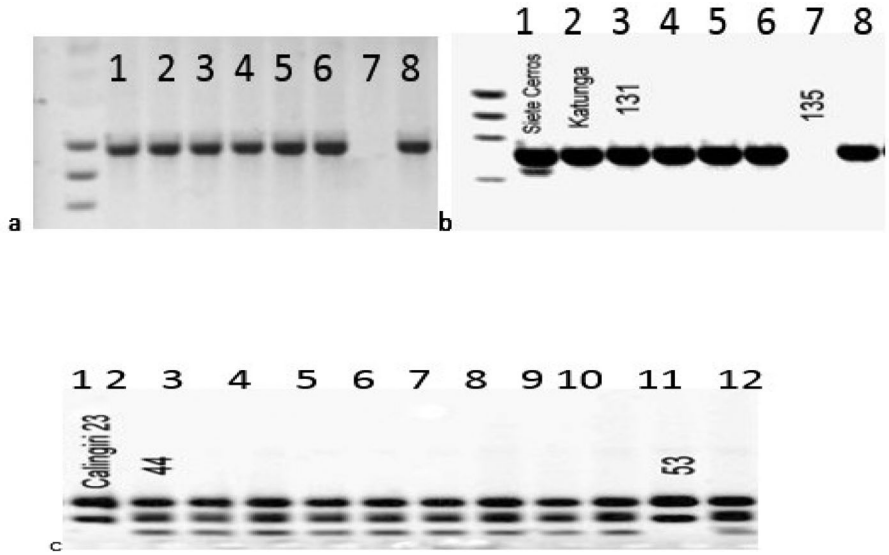


**Figure 2.** PCR analysis of Iranian bread-wheat landraces indicating the *Wx-D1b* and *Wx-B1b* (null mutations) (a) 1: Pavon (*Wx-D1a*); 2, Chinese Spring (*Wx-D1a*); 3, CWI 73213 (*Wx-D1a*); 4, CWI 7314 (*Wx-D1a*); 5, CWI 73215 (*Wx-D1a*); 6, CWI 73216 (*Wx-D1a*); 7, CWI 67665 (*Wx-D1b*); 8, CWI 67674 (*Wx-D1a*); (b) 1, Chinese Spring (*Wx-B1a*); 2, Katunga (*Wx-B1a*); 3, CWI 71829 (*Wx-B1a*); 4, CWI 71830 (*Wx-B1a*); 5, CWI 57655(*Wx-B1a*); 6, CWI57662 (*Wx-B1a*); 7, CWI 57684 *(Wx-B1b)*; 8, CWI 57692 (*Wx-B1a*); (c) 1, Calingiri 23 (*Wx-B1b*); 2, CWI 73213 (*Wx-B1a*); 3, CWI 7314 (*Wx-B1a*); 4, CWI 73215 *(Wx-B1a*); 5, CWI 73215 (*Wx-B1a*); 6, CWI 67665 (*Wx-B1a*); 7, CWI67674 (*Wx-B1a*); 8, CWI 67701 (*Wx-B1a*); 9, CWI 67703 (*Wx-B1a*); 10, CWI 67708 (*Wx-B1a*); 11, CWI 67747 (*Wx-B1b*); 12, CWI 67749 (*Wx-B1a*).

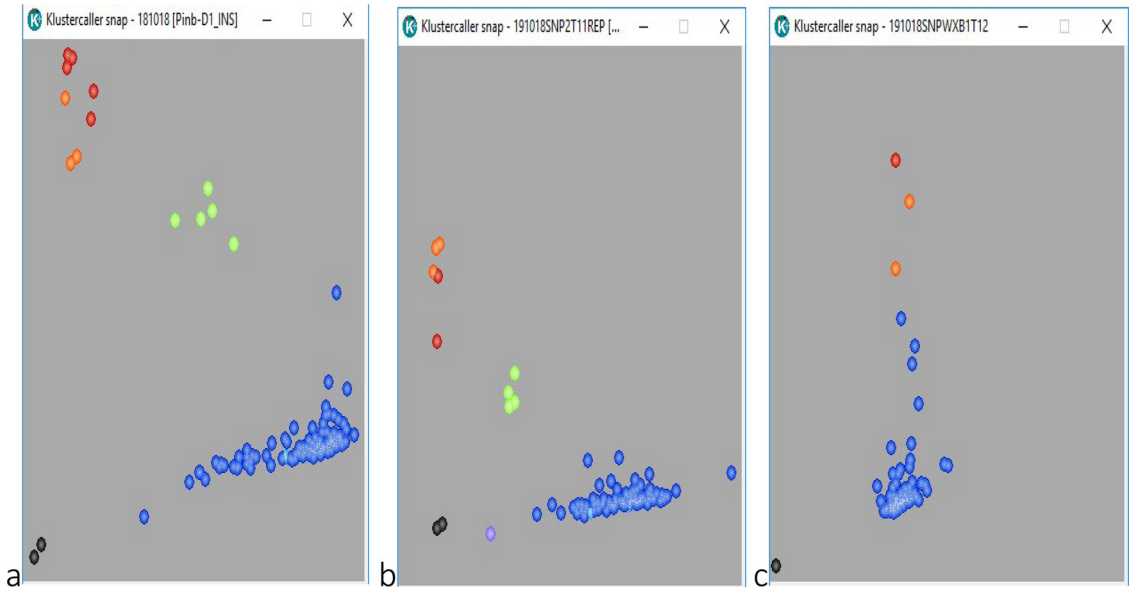


**Figure 3.** Codominant KASP markers developed in Iranian bead-wheat landraces for identifying (**a**) and (**b**) the *Pin-D* alleles. Black dots represent the non-template controls, blue dots represent the FAM-type or wild-type (soft) *pina-D1a*/*pinb-D1a*, green dots represent heterozygous alleles, red dots represent VIC or mutant-type (hard) *pina-D1b*/*pinb-D1b* and purple dots represent non amplification. (**c**) For *Wx-B* alleles with a dominant marker. Black dots represent the non-template controls; blue dots represent the FAM-type or wild-type *Wx-B1a* and red dots represent VIC or mutant-type *Wx-B1b*.

49, 57–62]. However, our result did not show differences in grain hardness between *Pina-D1b* and *Pinb-D1*null [63]. In this study, the findings may have been influenced by the following: 1) environmental conditions and grain size [14, 64–67]; however, Pomeranz *et al.* [68] showed that hardness-related measurements were more affected by genotype than by environment; 2) other minor genes [23, 69–71]; 3) the limited accuracy of NIR to predict PSI; 4) the limited number of accessions with different *Pin-D1* alleles and the

different genetic backgrounds of the accessions, which led to unidentifiably small differences owing to the presence of different Pin-D1 alleles [53]. Grain protein content is directly related to grain hardness [14, 58, 70, 72–74]. In the present study, the grain protein content was 15.7% on average. Thus, it was not surprising that few of the accessions showing hard grain phenotypes had protein contents of ∼15.43%. (data not shown).

However, Symes [75] reported that the correlation between protein content and hardness was positive in some cultivars and negative in other cultivars, which was corroborated by our results.

Starch composition is another parameter that affects the processing quality and is controlled by *Wx* genes (*Wx-A1*, *-B1* and *-D1*). All the accessions contained the allele *Wx-A1a* with the marker MAG264, which is similar to the results obtained by Liang *et al.* [76]. In our study, all the accessions contained the allele *Wx-D1a* with the marker MAG269, and only one accession (CWI 67665) indicated the presence of *Wx-D1b* (null mutation). However, the lack of *Wx-A1* or *Wx-D1* protein does not always lead to a significant decrease in the amylose content [34, 77], while the absence of *Wx-D1* has a greater effect compared with that of *Wx-A1* [78]. At the *Wx-B1* locus, using primers designed by Saito *et al.* [48] and Mclauchlan *et al.* [47], complete deletions of the gene existed and most accessions harbored the *Wx-B1a* allele, except two accessions (CWI67747 and CWI57684). Yamamori and Quynh [78] reported that Wx-B1b induced a lower amylose content, which was in agreement with an earlier finding [79]. They also analyzed the effects of *Wx* proteins and ranked the single null genotypes as *Wx-B1b* > *Wx-D1b* > *Wx-A1b*. Molecular characterizations of wheat waxy proteins and their effects on starch properties, as well as the detection of *Wx* null mutations, have been widely studied [47,48, 80–83], which is important for the identification waxy wheats in breeding programs [84]. The majority of KASP markers found in the CIMMYT used in this study were derived from the *Pin-D* analysis and were in agreement with the STS markers. KASP can be used for genotyping a wide range of species for various purposes that can help the development of grain quality.

Molecular markers for *Pin-D1* and *Wx* genes are imperative to wheat molecular breeding programs, because their different allelic compositions lead to different hardness levels [85] and amylose contents, which affect the end-use food quality of wheat grain [21]. Codominant and dominant markers are preferable for introducing null alleles into grain hardness and *Wx* proteins. The use of traditional gel-based PCR markers is time-consuming and results in a relatively low throughput, compared with more recently developed methods. The results of this study provide a deeper insight into the molecular and KASP markers that can be used to characterize grain hardness and *Wx* genes in bread wheat. Moreover, landraces offer an important genetic resource that can be used to improve modern varieties of wheat by means of introducing new alleles or a combination of genes.

## Conclusions

Waxy genes, including three null alleles, were found in the Iranian landrace collection described in this study. These alleles may be used to modify starch properties by adjusting of the standard amylose/amylopectin ratio, thereby enhancing the functionality of wheat flour doughs to improve traditional or novel wheat-based foods. In addition grain hardness is of paramount importance to wheat processors, end- users and those involved in wheat breeding and improvement. To this end, null alleles of puroindoline genes were determined in this collection of Iranian landraces. The results, therefore, provide evidence that these genetic materials are important sources of genetic diversity for developing wheat cultivars with improved grain textures.

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