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Allelic Variation of Puroindolines Genes in Iranian Common Wheat Landraces

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Abstract: Wheat is one of the most widely grown crops in the world. One of the traits that defines wheat quality is grain hardness, which is determined by puroindolines (PINA and PINB) proteins encoded with *Pina-D1* and *Pinb-D1* genes. In this study, the diversity of *Pina-D1* and *Pinb-D1* was evaluated in a collection of 271 Iranian common wheat (*Triticum aestivum* L. ssp. *aestivum*) landraces, whose kernels had previously been classified as hard or semi-hard based on PSI analysis. Three alleles previously described as associated with hard grain were detected in the collection: *Pinb-D1b* in 11 accessions, *Pinb-d1ab* in 175 accessions, and *Pinb-d1p* in 80 accessions. In addition, a novel allele tentatively named *Pinb-d1ak* was detected in *Pinb-D1* and was characterized by a change at position 140 of the deduced protein (cysteine/tyrosine). On average, the accessions with this allele showed a lower PSI value than the accessions with other *Pin* allele. This means that this novel allele may be associated with harder grains than other *Pin* alleles and could be used by breeding programs targeting different grain hardness levels. This study highlights the importance of conserving and characterizing wheat genetic resources that could be used as sources of genetic variability in breeding programs.

Keywords: wheat landraces; *Triticum aestivum*; grain hardness; puroindolines; genetic diversity

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1. Introduction

Wheat (*Triticum* sp.) is one of the three main crops grown around the world, with a field area of 219 million hectares that represents a quarter of total cereal production, with 760 million tons produced in 2020 [1].

In wheat, grain quality is one important factor because it determines the market value and its subsequent use. Wheat quality is determined by grain composition and the characteristics derived from it: hardness, gluten strength, and starch. Grain hardness is the most important single trait determining end-use quality [2], as it is related to the amount of damaged starch produced during the milling process. Since hard grain produces more damaged starch than soft grain, hard wheat is used for bread-making, which requires high water absorption for the correct development of fermented dough, while soft wheat is preferred for making cookies and pastries, which requires less hydrophilic flour, so that more water is available for the sugar to form a syrup, resulting in greater cookie spread [3].

In common wheat (*Triticum aestivum* L. ssp. *aestivum*), grain hardness is genetically controlled by two genes (*Pina-D1* and *Pinb-D1*) that codify for puroindolines (PINA and PINB, respectively). Puroindolines are small (~13 kDa), cysteine-rich, lipid-binding proteins consisting of 148 amino acids [4]. These proteins exhibit a tryptophan-rich domain, which consists of five tryptophan residues in PINA and three residues in PINB [2], and both PINA and PINB contain a backbone of 10 cysteine residues [5]. The *Pin-D1* genes are located at the hardness locus (*Ha*) in the distal end of the 5DS chromosome [6]. Both genes have a 447 bp coding sequence [7], and these sequences are 70.2% similar to each other [8]. The *Ha* locus includes also the *Gsp-1* gene (495 bp) that codes for the grain softness protein-1 [9].

The presence of *Pina-D1a* and *Pinb-D1a* alleles in common wheat is associated with a soft texture, while nucleotide changes in coding regions affecting the original protein or deletions of entire *Pin-D1* genes (null alleles) are correlated with a hard texture [5]. Since allopolyploidization resulting in durum wheat (*T. turgidum* ssp. *durum* (Desf.) Husn) caused the loss of the puroindolines genes in the A and B genomes, durum wheat lacks puroindolines genes, and its grains are very hard [5]. The *Pin-D1* genes' polymorphisms led to differences in the degree of hardness and in processing and end-use quality traits [10]. Several studies were conducted comparing the effects of the *Pina-D1b* and *Pinb-D1b* alleles, which are the two most common *Pin-D1* alleles with hard endosperm. Genotypes with *Pina-D1b*/*Pinb-D1a* showed harder kernels than those with *Pina-D1a/Pinb-D1b* [11]. In addition to this, the *Pinb-D1b* allele was associated with higher milling yield, higher dough extensibility, and better baking quality (higher loaf volume and better crumb) than the *Pina-D1b* allele [12]. These studies clearly illustrate the importance of the *Pin-D1* genes not only for hardness but also for processing and end-use quality.

Wheat landraces possess variability in different genes that are not present in modern cultivars [13]. For wheat quality improvement, this variability could be useful in generating materials with novel properties. In fact, several puroindoline alleles have been found in different collections of genetic resources worldwide. For example, the allele *Pinb-D1d* was first detected when researchers were analyzing *Pin-D1* genes of cultivars from northern Europe [14], and later the same allele was found in cultivars with Spanish origins [15]. Chinese landraces have been showed to carry alleles very rare in other germplasm pools, such as *Pina-D1r*, *Pina-D1s*, and *Pinb-D1p* [16] or *Pinb-D1l* [17]. Similarly, cultivars with origins in India were found to be good sources for the variability of the *Pin-D1* genes by Kumar et al. [18], who identified five novel alleles in materials from that country.

Iran is one of the main habitats of wheat ancestors and is therefore a reservoir of new alleles [19]. In the Seeds of Discovery project at CIMMYT (International Maize and Wheat Improvement Center, Mexico), 30,000 wheat accessions (including a collection of 6800 Iranian wheat landrace accessions [19]) were characterized for quality traits [20], including grain hardness, using the PSI method [21].

The objective of our study was to assess the diversity for the *Pina-D1* and *Pinb-D1* genes in a collection of 271 common wheat landraces from Iran, in order to detect new alleles of the puroindoline genes associated with different grain hardnesses.

2. Materials and Methods

2.1. Plant Materials

In this study, 271 Iranian common wheat landraces, which were provided by the CIMMYT's germplasm bank and known from a previous study to have hard and semi-hard grains, were used [20]. The hardness data of all of them were determined by near infrared spectroscopy (NIRS, Antaris II FT-Analyzer, Thermo Scientific, Waltham, MA, USA), calibrated on the basis of AACC methods [21] (Supplementary Table S1).

2.2. Genomic DNA Extraction

Genomic DNA was extracted from the young leaves of approximately two-week-old seedlings grown using the modified CTAB method described by Stacey and Isaac [22], and the samples were diluted to a final concentration of 20 ng/mL.

2.3. Amplification and Digestion of Pina-D1 and Pinb-D1 Genes

The *Pina-D1* gene was amplified with the primers designed by Lillemo et al. [23] (LIL1-Fw: 5′-CATCTATTCATCTCCACCTGC-3′ and LIL1-Rv: 5′-GTGACAGTTTATTAGCTAGTC-3′) and with the primers designed by Massa et al. [24] (MAS-Fw: 5′-GGTGTGGCCTCATCT CATCT-3′ and MAS-Rv: 5′-AAATGGAAGCTACATCACCAGT-3′). For the amplification of the *Pinb-D1* gene, we used the primers designed by Lillemo et al. [23] (LIL2-Fw: 5′-GAGCCTCAACCCATCTATTCATC-3′ and LIL2-Rv: 5′-CAAGGGTGATTTTATTCATAG-3′).

Each 15 mL reaction included 40 ng of DNA, 1.5 mM $MgCl_2$, 0.3 mM of each primer, 0.2 mM dNTPs, 3 µL of 5× PCR buffer, and 0.75 U of GoTaq® G2 Flexi DNA polymerase (Promega). The PCR conditions included an initial denaturation for 3 min at 94 °C followed by 35 subsequent cycles: 45 s at 94 °C, 1 min 30 s at 60 °C, and then 45 s at 72 °C for the primers LIL1 and LIL2; and 45 s at 94 °C, 30 s at 64 °C, and then 45 s at 72 °C for the primer MAS. After the 35 cycles, all reactions included a final extension of 5 min at 72 °C. The amplification products were fractionated on vertical PAGE gels in a discontinuous Tris-HCl buffer system (pH 6.8/8.8) with 8% (*w/v*, C: 1.28%) polyacrylamide concentration.

To detect some mutations already described in *Pina-D1* and *Pinb-D1* genes, the amplicons of these genes were digested with endonucleases or restriction enzymes. The *Pina-D1* amplicons were digested with *Msp*I (Biolabs) to detect the *Pina-D1m* allele [25] and with *Dde*I (Biolabs) to see if there were differences in the pattern when digesting the different accessions. The *Pinb-D1* amplicons were digested with *Bsr*BI (Biolabs) to detect the *Pinb-D1b* allele [26], with *Pvu*II (Biolabs) to detect the *Pinb-D1c* allele [14], with *Bst*XI (Biolabs) to detect the *Pinb-D1e* allele [26], with *Apa*I (Biolabs) to detect the *Pinb-d1ab* allele [26], and with *Pfl*MI (Biolabs) to detect the *Pinb-D1p* allele [27]. Digests were performed in a total volume of 10 µL, with 1 µL of buffer (1×), 0.25 U of enzyme, and 5 µL of PCR product for 2 h at 37 °C. The products resulting from this digestion were analyzed using electrophoresis on 10% polyacrylamide concentration gels (*w/v*; C: 3%).

2.4. Cloning of Pinb-D1 Genes

The *Pinb-D1* amplicons were then purified with SureClean Plus (Bioline Reagents, London, UK) and cloned into the vector pSpark TA Done (Canvax, Cordoba, Spain). The nucleotide sequences were obtained from three positive clones. These sequences were analyzed and compared with the common wheat sequences available in the NCBI database for *Pinb-D1a*: DQ363913 using the Geneious Pro software version 2020.2.4 (Biomatters Ltd., Auckland, New Zealand). To evaluate the impact of the allelic variations in the PINB protein's function, the potential effects of amino acid changes were analyzed with PROVEAN (Protein Variation Effect Analyzer) [28]. In PROVEAN (http://provean.jcvi.org, accesed on 1 June 2022), variants with values below −2.5 are predicted as deleterious, while values above −2.5 are considered neutral [28]. Finally, the novel sequence found in the current study is available from the Genbank database (NCBI ID: ON723901).

3. Results

3.1. Variation in Grain Hardness

A large set of Iranian common wheat landraces were evaluated previously for grain hardness using the PSI test [20]. For the current study, a subset of those accessions was selected based on their PSI values. The selected accessions showed an average PSI value of 47.14 ± 2.74% and values ranging between 40 and 50%. These values correspond to those of hard and semi-hard grains: 221 accessions showed a semi-hard grain texture ranging between 45 and 50%, while 50 accessions were classified as semi-hard grain texture since they ranged between 45 and 40% (Figure 1). According to the literature [5], these accessions should carry mutations in either *Pina-D1* or *Pinb-D1* that lead to their non-soft grain texture.

3.2. PCR Analysis of the Pina-D1 and Pinb-D1 Genes

The *Pina-D1* and *Pinb-D1* genes were amplified to detect possible differences in their size, together with the presence/absence of amplicons. For the *Pina-D1* gene using primer LIL1, all of the accessions produced a PCR product of 524 bp in size (Figure 2A). When the primer MAS was used, the results for *Pina-D1* amplification presented two amplicons (Figure 2B), one of them with 516 bp (*Pina-D1*) and the other with 564 bp that corresponded to the grain softness protein (*Gsp-1* gene). All accessions showed the expected amplification product of 597 bp for the *Pinb-D1* gene (Figure 2C). Therefore, no apparent polymorphism was detected in any of the accessions, and the amplicons seemed similar to the ones of cv. Chinese Spring (*Pina-D1a*, *Pinb-D1a*).

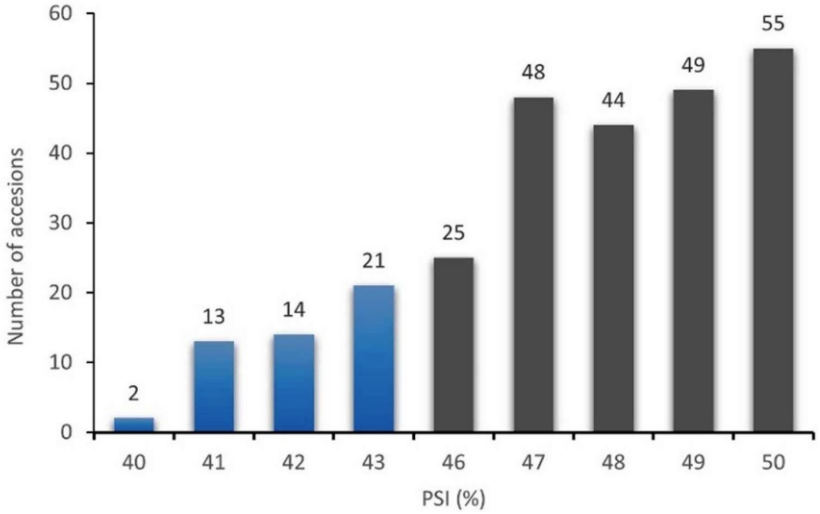

Figure 1. PSI frequency distribution of the accessions evaluated. Blue bars indicate hard grain accessions; grey bars correspond to semi-hard grain accessions.

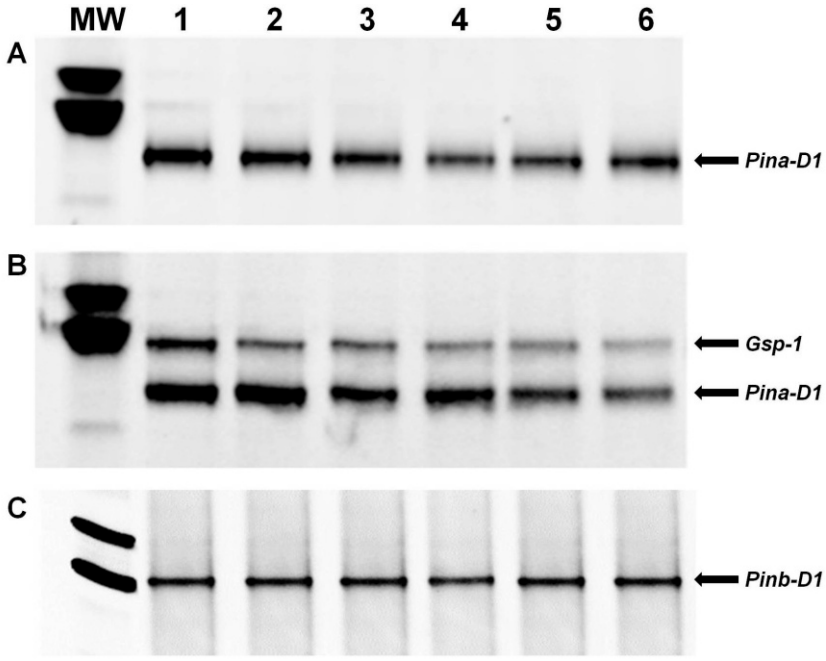

Figure 2. (**A**) *Pina-D1* amplification with primer LIL1; (**B**) *Pina-D1* amplification with primer MAS; (**C**) *Pinb-D1* amplification with primer LIL2. Lanes represent as follows: (**1**) cv. Chinese Spring (*Pina-D1a*/*Pinb-D1a*); (**2**) CWI 67429; (**3**) CWI 57816; (**4**) CWI 67483; (**5**) CWI 73046; and (**6**) CWI 72689.

3.3. Enzymatic Digestion and Sequencing of Pinb-D1 Alleles

Several specific restriction enzymes were used to digest the amplicons of *Pina-D1* and *Pinb-D1* in order to screen the Iranian landraces that were tested for the most common mutations for *Pina-D1* and *Pinb-D1*. The *Pina-D1* amplicons were digested with *Msp*I (used to detect the *Pina-D1m* allele), but no accession provided a positive result for this allele (Figure 3). Additionally, the *Pina-D1* amplicons were also digested with *Dde*I (used by Guzmán et al. [29] to detect polymorphism in *Pina-D1*), but again no polymorphism was detected (data not shown).

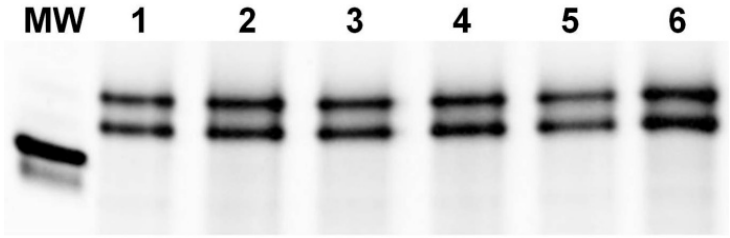

Figure 3. *Pina-D1* digestion with *Msp*I endonuclease. Lanes represent as follows: (**1**) cv. Chinese Spring *(Pina-D1a/Pinb-D1a*); (**2**) CWI 67429; (**3**) CWI 57816; (**4**) CWI 67483; (**5**) CWI 73046; and (**6**) CWI 72689.

However, several *Pinb-D1* alleles were found in our accessions using specific restriction enzymes (Figure 4). We found 11 accessions with the *Pinb-D1b* allele (positive for *Bsr*BI digestion), 80 accessions with *Pinb-d1p* (negative for *Pfl*MI digestion), and 175 accessions with the *Pinb-D1ab* allele (negative for *Apa*I digestion) (Table 1). The alleles *Pinb-D1c* and *Pinb-D1e* (digestions with *Pvu*II and *Bst*XI, respectively) were not found in any of our accessions (data not shown).

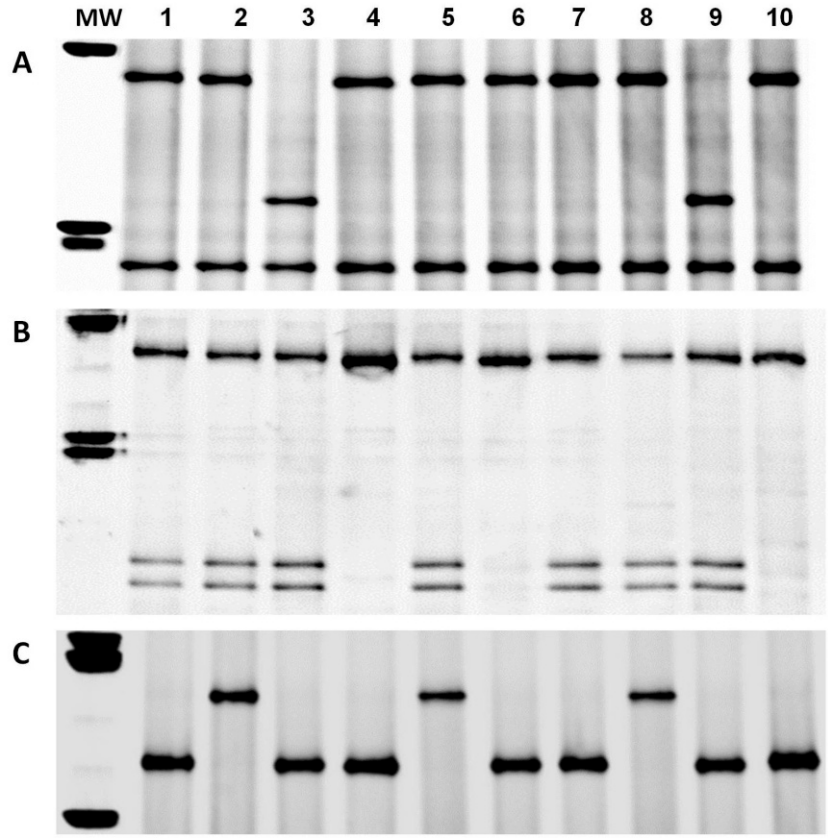

Figure 4. (**A**) *Pinb-D1* digestion with *Bsr*BI endonuclease; (**B**) *Pinb-D1* digestion with *Pfl*MI endonuclease; (**C**) *Pinb-D1* digestion with *Apa*I endonuclease. Lanes represent as follows: (**1**) cv. Chinese Spring *(Pinb-D1a*); (**2**) CWI 67429 (*Pinb-D1ab*); (**3**) CWI 57816 (*Pinb-D1b*); (**4**) CWI 67483 (*Pinb-D1p*); (**5**) CWI 73046 (*Pinb-D1ab*); (**6**) CWI 72689 (*Pinb-D1p*); (**7**) CWI 72663 (*Pinb-D1a*); (**8**) CWI 73075 *(Pinb-D1ab*); (**9**) CWI 66889 (*Pinb-D1b*); and (**10**) CWI 66921(*Pinb-D1p*).

Table 1. List of *Pinb-D1* alleles that were searched for specific restriction enzymes in our study.

Allele	Mutation	N	Reference
Pinb-D1b	Gly/Ser at position 46	11	[6,26]
Pinb-D1ab	Gln/Stop at position 382	175	[17,26]
Pinb-D1p	Lsy/Asn at position 210	80	[27,30]
Pinb-D1e	Trp/Stop at position 39	0	[26,31]
Pinb-D1c	Leu/Pro at position 60	0	[14]

To detect more variability in the *Pinb-D1* gene, the remaining five accessions that did not show any differences when compared with those of cv. Chinese Spring in the above-mentioned analysis were selected for the sequencing of their *Pinb-D1* gene. The sequences obtained were compared with the sequence of the *Pinb-D1a* allele of cv. Chinese Spring (NCBI ID: DQ363913). All these accessions showed a G/A transition, resulting in a codon change at position 419 (TGC → TAG). According to a BLAST analysis performed and the literature reviewed, this mutation was not reported previously, and therefore we consider this allele to be novel. Following the order of the Wheat Gene Catalogue, we tentatively proposed to name this allele *Pinb-D1ak*. In addition, the results obtained with the restriction enzymes were corroborated by sequencing the *Pinb-D1* gene in few of the accessions that resulted in a positive for other alleles (*Pinb-D1p* and *Pinb-D1ab*). As all the accessions used in the study showed a mutation in *Pinb-D1* gene that explained their hard grain texture, it was assumed that they carried the *Pina-D1a* allele for *Pina-D1* gene.

3.4. Amino Acid Sequence Analysis and Relationship with Grain Hardness

Both PIN proteins are synthesized as precursors or preproteins and contain four domains: a signal-peptide, N-terminal domain, mature protein, and C-terminal domain [7]. Another distinctive feature of these proteins is the presence of a highly conservative cysteine backbone made up of ten cysteines and a tryptophan-rich domain. In the novel allele identified (*Pinb-D1ak*), which causes an amino acid change from cysteine to tyrosine at position 140 of the amino acid sequence, the cysteine change coincided with the tenth cysteine of the cysteine backbone of the PINB protein (Figure 5). In addition to this, the PROVEAN score obtained for the *Pinb-D1ak* allele (−4.2) indicated that this mutation likely has a strong effect on the protein function.

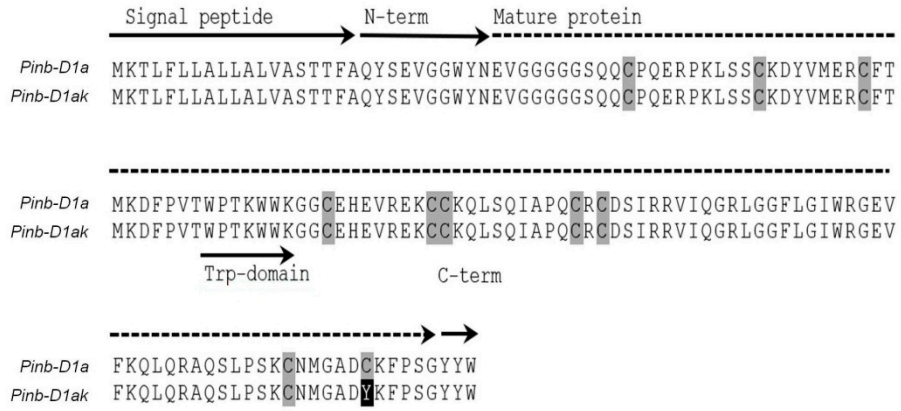

Figure 5. PINB-D1 protein structure and Trp-domain. The ten cysteine residues are marked in grey. Amino acid change C140Y for *Pinb-D1ak* allele is indicated in black.

The relationship between the grain hardness of the accessions with the new *Pinb-D1ak* allele and the rest of the accessions identified with the *Pinb-D1b*, *Pinb-D1ab*, and *Pinb-D1p* alleles was studied by analyzing the mean PSI of the group of accessions that had each allele. It was observed that the accessions with the *Pinb-d1ak* allele had a mean PSI value of 44.2 ± 2.93, slightly lower than that of the rest of the accessions groups with the *Pinb-D1b*, *Pinb-D1ab*, and *Pinb-D1p* alleles with mean PSI values of 45.91 ± 3.58, 46.91 ± 2.77, and

47.11 ± 2.64, respectively (Table 2). Therefore, it was observed that the accessions with the *Pinb-d1ak* allele had harder grains than those of the accessions with other mutations, as lower values of the PSI (%) correspond to harder grains [7].

Table 2. Allelic composition for puroindoline genes and the PSI values of the lines evaluated.

Puroindoline Composition	N	PSI (% ± s.d.)	Range
Pina-D1a/Pinb-D1b	11	45.91 ± 3.58 ^{AB*}	40–50
Pina-D1a/Pinb-D1ab	175	47.13 ± 2.70 ^{A}	40–50
Pina-D1a/Pinb-D1p	80	47.48 ± 2.64 ^{A}	41–50
Pina-D1a/Pinb-D1ak	5	44.20 ± 2.93 ^{B}	41–49

s.d.: Standard deviation; * letters indicate significantly ($p < 0.05$) different means groups.

4. Discussion

Landraces and old cultivars possess variability for different genes that may not be present in modern cultivars, and that knowledge could be useful for modern breeding programs to develop materials with novel differential properties. Because of this characteristic, efforts are being made to identify useful novel variations among the vast wheat genetic resources stored in germplasm banks. In this regard, the CIMMYT's wheat germplasm bank holds a huge collection (more than 100,000 accessions) that is being characterized by different means [14,32] in order to possibly use its unexploited variation for the genetic improvement of the breeding program. Within the CIMMYT's collection, there is a large set (6800 accessions) of landraces with Iranian origin since Iran is one of the centers of diversity for the common wheat. This set of materials has shown great genetic diversity [20], including interesting variations for grain quality traits related to both end-use and nutritional properties [33].

In this study, the grain texture of a set of Iranian bread wheat landraces was analyzed and characterized at the molecular level for puroindoline composition. Since grain hardness is one of the most important wheat quality traits, its genetic control has been extensively studied, and a lot of alleles of the two puroindoline genes involved (*Pina-D1* and *Pinb-D1*) have been identified. The *Pina-D1a* and *Pinb-D1a* alleles are associated with a soft grain texture [6], while most of the other described alleles of *Pina-D1* and *Pinb-D1* are associated with a hard grain texture. The variability of these genes was studied before in germplasm collections of diverse origins. For example, Cane et al. [34] examined the polymorphism of these genes in Australian wheat cultivars; Ayala et al. [15] screened Mexican landraces, finding three different *Pin-D1* alleles leading to hard grain; Ma et al. [16] characterized a large collection, mainly from China but with some cultivars from other countries (USA, Europe, Japan, etc.), to find six and nine different *Pina-D1* and *Pinb-D1* alleles, respectively. However, Iranian materials have been not studied in detail for the variability of these important genes. In our study, all the accessions were previously evaluated for grain hardness and had either hard or semi-hard grains. This indicated that the Iranian landraces selected for the current study likely carry mutations in the puroindoline genes that lead to a loss of function in their respective puroindoline proteins. Therefore, the probability of detecting mutations for the *Pina-D1* or *Pinb-D1* genes was rather high.

First, using specific restriction enzymes, we searched different alleles that were previously described to cause changes in the PINA or PINB protein sequence or their absence and lead to hard endosperm. The *Pinb-D1b* allele was found, which consists of a single nucleotide change at position 46 in the *Pinb-D1* sequence that confers a glycine to serine change in the expressed protein. This change in the sequence resides near the tryptophan-rich domain and probably changes the secondary or tertiary structure of the protein, leading to hard endosperm [6]. The *Pinb-D1b* allele was detected in 11 accessions, which corresponded to a frequency of 4% of the total of our samples. This allele was found in different studies in landraces from different countries such as Spain [35], Mexico [15], and China [10,27] with a frequency similar to that of our study. However, in other collections such as in a study of landraces from Poland [36], *Pinb-D1b* had a frequency of 21% of the total, which was much

higher than the frequency of our study. It was also very frequent in cultivars from Norway, Sweden, and Finland [14]. Therefore, we deduced that *Pinb-d1b* is widely distributed in America, Asia, and Europe, although in Europe the frequencies of *Pinb-D1b* in landraces are higher than in other regions.

Another allele found in our materials was *Pinb-d1ab*, which has a transition (C/T) at the 382 position, leading to the appearance of an early stop codon in the deduced mature protein (Gln → Stop) [17,21]. The presence of this premature stop codon causes the synthesis of a non-functional PINB protein, which explains the hard grain in genotypes with this mutation. This mutation is very similar to the ones present in *Pinb-D1f*, *Pinb-D1e*, and *Pinb-D1g* alleles [31,36]. These mutations lead to an increase in grain hardness by overriding the correct expression of the PINB protein through the appearance of premature stop codons in other regions of the gene. The *Pinb-d1ab* allele was detected in 175 accessions, equivalent to a frequency of 64.6% of our total collection. The *Pinb-d1ab* allele was first discovered in two accessions from Afghanistan [17]. Later, it was detected in landraces from China (in 19.1% of the landraces studied) [26] and from Pakistan (in 1% of the genotypes) [37]. Therefore, it seems that both the origin and distribution of the *Pinb-d1ab* allele is exclusive to the Asian continent.

The *Pinb-D1p* allele was also found in our collection; this allele consists of an adenine deletion at the 210 position, resulting in a lysine to asparagine change and then a premature stop codon in the mature protein [30]. The *Pinb-D1u* [10] and *Pina-D1l* [38] alleles have similar mutations, characterized by the deletion of a nucleotide in their sequence. The *Pinb-D1p* allele was found in 80 accessions, resulting in a frequency of 29.5% of the total of our collection. This frequency is lower than in the study of wheat landraces from Shandong (China), in which the frequency of the *Pinb-D1p* allele was 59.6% [27]. However, in other screenings with accessions from Pakistan and Afghanistan, this allele showed low frequency [17]. These studies indicate that this allele may have spread along the "Silk Road". Therefore, it appears that its distribution is only across the Asian continent, as in the case of *Pinb-d1ab*.

Finally, the sequencing of the *Pinb-D1* gene in the remaining accessions that did not show any of the mutations described above allowed for the detection of a novel allele (*Pinb-D1ak*) that had not been previously described. This allele caused a nucleotide change that generated a change from cysteine to tyrosine at the 140 position of the amino acid sequence. Several studies have shown that the conservation of domains in the PINA and PINB proteins is very important for the conservation of the soft texture of the grain [39,40], for which the backbone of cysteines (whose function is the stabilization of the structure of the puroindolines) is essential [41]. In this sense, it is understood that the *Pinb-D1ak* allele has a large effect on protein functionality according to the PROVEAN score obtained, because as previously mentioned, this allele causes a change in the tenth cysteine of the cysteine backbone. There are other alleles described with similar characteristics as they have changes affecting the cysteine backbone, such as *Pinb-D1x*, which also has an amino acid change (Cys57 → Tyr) [42]; in general, amino acid changes affecting the cysteine backbone are not very frequent. Other alleles in which a single amino acid change in the protein leads to an increase in grain hardness are *Pinb-D1d* [14] and *Pinb-D1q* [43]. In these cases, both alleles have an amino acid change within the tryptophan-rich domain: in *Pinb-D1d*, there is a change from tryptophan-44 to arginine, and in *Pinb-D1q*, there is a change from tryptophan-44 to leucine. All these alleles are particularly interesting since they encode a protein that could have a different functionality, which may lead to intermediate grain hardness levels between the hard endosperm caused by the *Pin-D1* null alleles and the soft endosperm associated with the *Pin-D1a* alleles. In fact, several studies showed that genotypes with the *Pina-D1b* allele (null) are harder than genotypes with the *Pinb-D1b* allele (Gly46 → Ser) [11,12]. In the case of the novel allele *Pinb-D1ak* found in this study, it was preliminary associated with a slightly harder grain texture than that of the other alleles reported; further studies with more balanced populations in terms of the frequency of each

Pin-D1 allele or with near isogenic lines are necessary to assess the level of grain hardness associated with this novel allele.

5. Conclusions

Grain hardness is of utmost importance for wheat processors, end-users, and those involved in wheat breeding and improvement. The materials used in this study showed remarkable variability for the puroindoline genes. One of the four mutations found in *Pinb-D1* had not been previously described, was considered novel, and was named *Pinb-D1ak*. This mutation is in the tenth cysteine of the protein backbone, which is a highly conserved region. On average, accessions with the *Pinb-D1ak* allele showed harder grains than accessions with other *Pinb-D1* alleles did. Thus, this new allele may be associated with harder kernels than other *Pin* alleles and could be used by breeding programs targeting different levels of kernel hardness. This study highlights the importance of conserving and characterizing wheat genetic resources that could be used as sources of genetic variability in breeding programs

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12081196/s1, Table S1: Samples used in this study.

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