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The Variability of Puroindoline-Encoding Alleles and Their Influence on Grain Hardness in Modern Wheat Cultivars Cultivated in Poland, Breeding Lines and Polish Old Landraces (*Triticum aestivum* L.)

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Abstract: Wheat (*Triticum aestivum* L.) grain hardness is determined mainly by variations in puroindoline genes (*Pina-D1* and *Pinb-D1*), which are located on the short arm of chromosome 5D. This trait has a direct effect on the technological properties of the flour and the final product quality. The objective of the study was to analyze the mutation frequency in both *Pin* genes and their influence on grain hardness in 118 modern bread wheat cultivars and breeding lines cultivated in Poland, and 80 landraces from Poland. The PCR products containing the *Pin* gene coding sequences were sequenced by the Sanger method. Based on detected the SNPs (single-nucleotide polymorphisms) we designed CAPS (cleaved amplified polymorphic sequence) markers for the fast screening of *Pinb* alleles in a large number of genotypes. All analyzed cultivars, breeding lines, and landraces possess the wild-type *Pina-D1a* allele. Allelic variation was observed within the *Pinb* gene. The most frequently occurring allele in modern wheat cultivars and breeding lines (over 50%) was *Pinb-D1b*. The contribution of the remaining alleles (*Pinb-D1a*, *Pinb-D1c*, and *Pinb-D1d*) was much less (approx. 15% each). In landraces, the most frequent allele was *Pinb-D1a* (over 70%), followed by *Pinb-D1b* (21% frequency). *Pinb-D1c* and *Pinb-D1g* were found in individual varieties. SKCS (single-kernel characterization system) analysis revealed that grain hardness was strictly connected with *Pinb* gene allelic variation in most tested cultivars. The mean grain hardness values were significantly greater in cultivars with mutant *Pinb* variants as compared to those with the wild-type *Pinb-D1a* allele. Based on grain hardness measured by SKCS, we classified the analyzed cultivars and lines into different classes according to a previously proposed classification system.

Keywords: grain hardness; PIN; kernel texture; triticum; wheat; SKCS

1. Introduction

In Europe, common wheat (*Triticum aestivum* L.) is cultivated on almost 61 million hectares of land, and, in Poland, on 2.416 million hectares, which accounts for about 31% of all cereals cultivated in Poland [1]. Wheat is an important component of food and feed. Wheat grains, depending on their technological properties, are used for various food products [2]. Grain hardness, one of the most important technological traits, is considered as significant as gluten protein composition [3]. It has a direct effect on the grinding and baking characteristics, flour particle sizes, and water absorption

ability, which represent crucial technological flour properties [3,4]. In common wheat, grain hardness is primarily controlled by two puroindoline genes, *Pina* and *Pinb*. Both genes are intronless, with a 447 bp coding sequence and are located in locus *Ha* on the short arm of chromosome 5D [5]. The *Pin* gene coding sequences are 70.2% similar. In the course of wheat evolution, puroindoline genes located on chromosomes 5A and 5B were lost after wheat ancestor polyploidization [6]. *Pina* and *Pinb* encode the following puroindoline proteins: puroindoline a (PINA), and puroindoline b (PINB). Puroindolines are small (~13 kDa), cysteine-rich, lipid-binding proteins consisting of 148 amino acids [7,8]. PIN proteins exhibit a tryptophan-rich domain, which consists of five tryptophan residues in PINA and three residues in PINB [5]. Increased grain hardness is the result of mutations within one or both wild-type *Pin* alleles, which cause amino acid sequence changes in puroindoline proteins [9]. Thus far, 30 *Pina* alleles and 26 *Pinb* alleles have been described in different wheat species [10–13]. In most cases, the differences between wild-type and mutant alleles are single nucleotide polymorphisms (SNPs), such as in *Pinb-D1b* and *Pinb-D1c*, or one base deletion, like in *Pinb-D1p* and *Pinb-D1u*. Allele *Pinb-D1b*, originally described by Giroux and Morris [14], is characterized by guanine to adenine substitution at position 223, resulting in glycine to serine substitution in PINB at position 46 and changes in the tryptophan-rich domain (TRD). It is the most frequent *Pin* gene mutation among all domesticated wheat cultivars. *Pinb-D1c* and *Pinb-D1d* are the second most frequent alleles found in common wheat cultivars [11]. *Pinb-D1c* is characterized by thymine to cytosine substitution at position 266, leading to leucine to proline change at position 60 [15]. In *Pinb-D1d*, thymine to adenine substitution at position 217 causes tryptophan to arginine change at position 44 and leads to changes in the TRD [15]. An example of the change in reading frame is the allele *Pinb-D1g*, where a single nucleotide change at position 255 (cytosine to adenine substitution) causes a cysteine to stop codon change at position 56 [16]. An example of a SNP which does not change the amino acid sequence is the *Pinb-D1ae* allele, in which thymine is substituted by adenine at position 93 [17]. Less common alleles possess more than one SNP, e.g., two SNPs in *Pinb-D1v* [18] or one SNP and one base deletion in *Pinb-D1s* [19]. *Pina-D1b* is a null mutation resulting from the deletion of a 15.3 Kb chromosome fragment [20,21].

Genetic diversity and grain hardness allelic variation were studied in Australian commercial wheat cultivars [22], the Watkins collection [23], and bread wheat landraces from Andalusia [24]. Additionally, the trait is also easily genetically engineered [25–27]. Both *Pin* genes are considered the main grain hardness determinants. TRD is the most important domain of PIN proteins because it is responsible for their lipid-binding properties. Changes in TRD caused by SNP in *Pinb-D1b* and *Pinb-D1d* alleles result in decreased affinity to anionic phospholipids and altered interaction between PINA and PINB proteins [28,29]. TRD is comprised of five tryptophan (Trp) residues in PINA and three Trp residues in PINB [5]. Apart from puroindoline-encoding genes, very similar genes showing over 70% similarity with the *Pinb* nucleotide sequence have been identified. They are categorized as *Pinb-2* variants and are located on chromosomes 7A, 7B, and 7C [30–33]. However, further studies clearly demonstrated that the *Pinb-2* variants expression levels were very low and these genes had no significant effect on grain hardness [31,34].

In this study, we analyzed allelic variation in *Pin* genes in collection of hexaploid wheat cultivated in Poland, including Polish and European cultivars, as well as in Polish breeding lines and landraces, and we determined how this variation affected grain hardness. The cleaved amplified polymorphic sequence (CAPS) markers adapted to the screening of *Pinb* alleles proved to be useful.

2. Materials and Methods

2.1. Plant Material

The plant material consisted of 67 wheat cultivars cultivated in Poland in 2015–2018, and 51 breeding lines from Polish breeding companies, and all 80 landraces collected in Poland between 1960 and 1976, and deposited in the National Centre for Plant Genetic Resources (KCRZG) of the Plant Breeding and Acclimatization Institute – National Research Institute. Plants were grown during

2018–19 in a greenhouse, with eight plants in a twelve liter pot using standard agronomic practices including preventative spraying with plant protection products. The full list of wheat genotypes is presented in Supplementary Tables S1 and S2.

2.2. DNA Extraction

The seedlings were grown in beakers with 4 mm glass balls and autoclaved tap water, in darkness at 23 °C. Genomic DNA was extracted from coleoptile fragments and leaves of six-day old seedlings using a modified cetrimonium bromide (CTAB) method devised by Murray and Thompson [35]. DNA was extracted from the pooled tissue samples of at least six individuals from the same variety. The DNA purity and concentration were checked using a Nanodrop 1000 (NanoDrop Technologies) and diluted to 50 ng/µL for further PCR analysis.

2.3. Pina and Pinb PCR Amplification and Sanger Sequencing

Both alleles of the *Pin* genes were sequenced in all examined (198) wheat genotypes. Two primer pairs were used for PCR amplification and sequencing: *Pina* forward: 5′-CATCTATTCATCTCCA CCTGC-3′; reverse: 5′-GTGACAGTTTATTAGCTAGTC-3′ (product length—524 bp); *Pinb* forward: 5′-GAGCCTCAACCCATCTATTCATC-3′; reverse: 5′-CAAGGGTGA TTTTATTCATAG-3′ (product length—595 bp) [36]. The PCR mix contained: 100 ng DNA, 0.5 µM of each primer, 200 µM deoxynucleotide triphosphates (dNTPs) mix, 0.4 U Phusion Hot Start II DNA Polymerase (Thermo Scientific), 4 µL 5× Phusion HF buffer, and PCR-grade water up for a 20 µL final volume. PCR was performed under the following conditions: initial denaturation at 98 °C for 30 s, followed by 36 cycles consisting of denaturation at 98 °C for 10 s, annealing at 58 °C (for both primer pairs) for 30 s, DNA elongation at 72 °C for 15 s, and, after 36 cycles, the final extension step at 72 °C for 8 min.

The amplification products were purified using the GeneJET PCR Purification Kit (Thermo Scientific), according to the manufacturer's instructions, and then sequenced by the Sanger method using the same primers that were used for amplification. The samples were sequenced by Genomed S.A. Sequences, which were aligned using MegAlgin (DNASTAR) software.

2.4. CAPS Analysis

The *Pinb* gene primer sequences for amplification were as follows: forward: 5′-ATGAAGACCT TATTCC TCCTA-3′; reverse: 5′-AGTAATAGCCACTAGGGAACTT-3′ (product length—442 bp) [5]. The PCR mix was composed of: 100 ng DNA, 0.5 µM of each primer, 200 µM dNTP mix, 0.4 U Phusion Hot Start II DNA Polymerase (Thermo Scientific), 4 µL 5× Phusion HF buffer, and PCR-grade water up to a final volume of 20 µL. PCR was performed under the following conditions: initial denaturation at 98 °C for 30 s followed by 36 cycles consisting of denaturation at 98 °C for 10 s, annealing at 53 °C for 30 s, DNA elongation at 72 °C for 15 s, and, after 36 cycles, the final extension step at 72 °C for 8 min. PCR product was digested with restriction enzymes selected using the SNP2CAPS program [37] to confirm the sequencing results. The digest reaction was composed of: 5 µL PCR product, 0.7 µL restriction enzyme, 2 µL 10× compatible buffer, and PCR grade water up to a final reaction volume of 20 µL. Samples were incubated at 37 °C in a water bath for 3 h. Digested fragments were separated on a 2.5% agarose gel by electrophoresis in SB buffer (Brody and Kern, 2004). *Mbi*I (Thermo Scientific) was used to confirm *Pinb-D1b*, *Pvu*II (Thermo Scientific) for *Pinb-D1c*, and *Mnl*I (Thermo Scientific) for *Pinb-D1d*. The amplicons containing *Pinb-D1a*, *Pinb-D1c* and *Pinb-D1d* alleles cut by *MbiI* gave two fragments, which were—318 and 129 bp long. The amplicon with *Pinb-D1b* cut with the same enzyme gave three fragments, of 223, 129 and 95 bp. Amplicons with *Pinb-D1a*, *Pinb-D1b* and *Pinb-D1d* digested with *PvuII* gave two fragments, which were—264 and 183 bp long, and the amplicon with *Pinb-D1c*, that was not cut with this enzyme had a fragment that was 442 bp long. Digestion of amplicons with *Pinb-D1a*, *Pinb-D1b*, and *Pinb-D1c* with *MnlI* led to nine fragments, which had the following lengths—137, 112, 66, 45, 29, 25, 20, 5 and 3 bp. Digestion with the same enzyme of *Pinb-D1d* amplicon gave ten fragments, which had the following lengths—115,112, 66, 45, 29, 25, 22, 20, 5, and 3 bp.

2.5. Grain Hardness Measurement

The grain hardness was measured using the Single Kernel Characterization System (SKCS) 4100 (Perten Instruments). Single kernel characterization was measured according to Approved Method 55–31.01 [38]. The hardness of each wheat kernel was determined by the instrumental measurement of the force required to crush the kernel. The SKCS instrument was calibrated to compute the hardness index including the weight, moisture, and diameter of each kernel. For each sample, 300 individual kernels were analyzed.

2.6. Percentage of Protein, Starch, and Wet Gluten

The percentage of protein, starch, and wet gluten was measured using the Infratec Nova Grain Analyser (Foss). Analyses were performed for landraces in two independent replicates. Samples of 30 g grains were used.

2.7. Statistical Analysis

Statistical analysis was performed using the R v. 3.6.2 and the FSA v. 0.8.27 package [39]. The normality distribution was verified by the Shapiro—Wilk test. The Kruskal—Wallis test was performed to compare variable distributions of *Pinb* allele groups and hardness index. The Dunn test was performed in order to establish which groups were different from each other. Pearson correlationa between hardness and protein or starch or gluten content were calculated separately for landraces with the wild-type *Pinb* alleles and mutant alleles, as well as for all landraces, regardless of their allelic status. The chart of hardness index ranges was performed using gglot2 v. 3.2.1 package [40].

3. Results

The allelic variation of *Pina* and *Pinb* was determined by Sanger sequencing. All tested cultivars, breeding lines, and landraces had the wild-type allele of the *Pina* gene (*Pina-D1a*). The frequencies of *Pinb-D1* alleles are shown in Table 1. The most frequent allele among modern cultivars and breeding lines was *Pinb-D1b* (57%). The wild type *Pinb-D1a* allele occurred in 19 lines (16%) and the frequencies of the remaining alleles, *Pinb-D1c* and *Pinb-D1d*, were at a similar level (12% and 15%, respectively).

Table 1. The *Pinb* allele frequency in the tested genotypes and mean hardness index (HI) in each allele group.

Pinb Allele	No. Cultivars (%)	Mean HI (SD)
Cultivars and Breeding Lines		
Pinb-D1a	19 (16%)	31.31 (±19.50)
Pinb-D1b	67 (57%)	59.36 (±11.73)
Pinb-D1c	14 (12%)	63.70 (±10.43)
Pinb-D1d	18 (15%)	59.08 (±13.80)
Landraces		
Pinb-D1a	57 (71%)	23.34 (±6.09)
Pinb-D1b	21 (26%)	49.23 (±10.97)
Pinb-D1c	1 (1%)	65.80
Pinb-D1g	1 (1%)	60.79

In landraces, the *Pinb* frequencies were different to modern cultivars and breeding lines. Over 71% had the *Pinb-D1a* allele, while 26% (21) had the *Pinb-D1b* allele. *Pinb-D1c* and *Pinb-D1g* were each found in one tested landrace. Alignment of the nucleotide and amino acid sequences of *Pinb* alleles with the marked mutations is shown in Figure 1, and chromatogram fragments of five alleles found in tested wheat genotypes are shown in Supplementary Figure S1.

a

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Pinb-D1a   ATGAAGACCTTATTCCTCCTAGCTCTCCTTGCTCTTGTAGCGAGCACAACCTTCGCGCAATACTCAGAAGTTGGCGGCTGGTACAATGAAGTTGGCGGAGGAGGTGGTTCTCAACAATGT  120
Pinb-D1b   ------------------------------------------------------------------------------------------------------------------------  120
Pinb-D1c   ------------------------------------------------------------------------------------------------------------------------  120
Pinb-D1d   ------------------------------------------------------------------------------------------------------------------------  120
Pinb-D1g   ------------------------------------------------------------------------------------------------------------------------  120

Pinb-D1a   CCGCAGGAGCGGCCGAAGCTAAGCTCTTGCAAGGATTACGTGATGGAGCGATGTTTCACAATGAAGGATTTTCCAGTCACCTGGCCCACAAAATGGTGGAAGGGCGGCTGTGAGCATGAG  240
Pinb-D1b   ------------------------------------------------------------------------------------------------A-----------------  240
Pinb-D1c   ------------------------------------------------------------------------------------------------------------------------  240
Pinb-D1d   ------------------------------------------------------------------------------------------------A-----------------  240
Pinb-D1g   ------------------------------------------------------------------------------------------------------------------------  240

Pinb-D1a   GTTCGGGAGAAGTGCTGCAAGCAGCTGAGCCAGATAGCACCACAATGTCGCTGTGATTCTATCCGGCGAGTGATCCAAGGCAGGCTCGGTGGCTTCTTGGGCATTTGGCGAGGTGAGGTA  360
Pinb-D1b   ------------------------------------------------------------------------------------------------------------------------  360
Pinb-D1c   ------------------------C-----------------------------------------------------------------------------------------------  360
Pinb-D1d   ------------------------------------------------------------------------------------------------------------------------  360
Pinb-D1g   ----------A-------------------------------------------------------------------------------------------------------------  360

Pinb-D1a   TTCAAACAACTTCAGAGGGCCCAGAGCCTCCCCTCAAAGTGCAACATGGGCGCCGACTGCAAGTTCCCTAGTGGCTATTACTGGTGA 447
Pinb-D1b   ------------------------------------------------------------------------------------------ 447
Pinb-D1c   ------------------------------------------------------------------------------------------ 447
Pinb-D1d   ------------------------------------------------------------------------------------------ 447
Pinb-D1g   ------------------------------------------------------------------------------------------ 447
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b

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Pinb-D1a   MKTLFLLALLALVASTTFAQYSEVGGWYNEVGGGGGSQQCPQERPKLSSCKDYVMERCFTMKDFPVTWPTKWWKGGCEHEVREKCCKQLSQIAPQCRCDSIRRVIQGRLGGFLGIWRGEV 120
Pinb-D1b   ************************************************************************S*********************************************** 120
Pinb-D1c   ************************************************************************P*********************************************** 120
Pinb-D1d   ************************************************************************R*********************************************** 120
Pinb-D1g   ***************************************************************************----------------------------------------- 120

Pinb-D1a   FKQLQRAQSLPSKCNMGADCKFPSGYYW  148
Pinb-D1b   ****************************  148
Pinb-D1c   ****************************  148
Pinb-D1d   ****************************  148
Pinb-D1g   ----------------------------  148
```

Figure 1. (**a**) Alignment of the nucleotide and (**b**) amino acid sequences of *Pinb* alleles detected in the tested cultivars.

Next, we examined whether the mutations in the *Pinb* coding sequence could be detected by CAPS analysis. Restriction enzyme digestion of the amplified full length *Pinb* coding sequences produced allele-specific fragment patterns, which were unique for each enzyme. The *Pinb-D1b*, *Pinb-D1c*, and *Pinb-D1d* alleles can be distinguished from the wild-type *Pinb-D1a* allele by restriction analysis with *Mbi*I, *Pvu*II, and *Mnl*I, respectively (Figure 2). The SKCS hardness values were grouped by each *Pinb* allele and by genotype (modern vs. landraces) (Figure 3). In modern cultivars and breeding lines the hardness index for each allele group ranged as follows: for *Pinb-D1a*, from 6 to 57; for *Pinb-D1b*, from 35 to 80; for *Pinb-D1c*, from 42 to 77; for *Pinb-D1d* from 40 to 82. In landraces, the hardness index for the *Pinb-D1a* group ranged from 9 to 37 and for *Pinb-D1b* from 18 to 63. The landrace with *Pinb-D1c* had a hardness index of 66, and another with *Pinb-D1g* had a hardness index of 61 (Supplementary Tables S1 and S2).

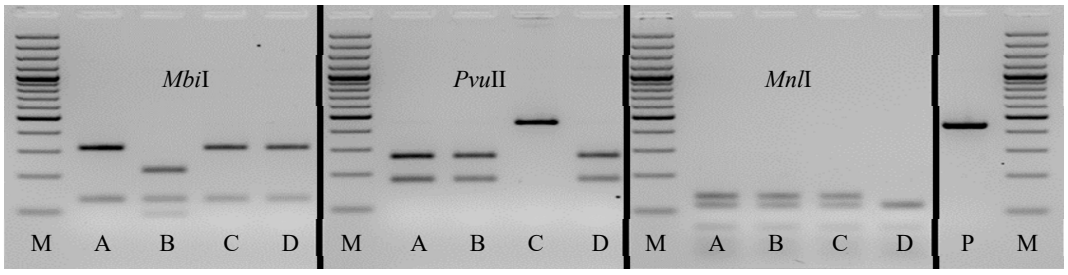

Figure 2. Examples of cleaved amplified polymorphic sequence (CAPS) analysis for detection of different *Pinb* alleles. M—molecular weight marker; A—*Pinb-D1a*; B—*Pinb-D1b*; C—*Pinb-D1c*; D—*Pinb-D1d*; P—undigested 447 bp PCR product.

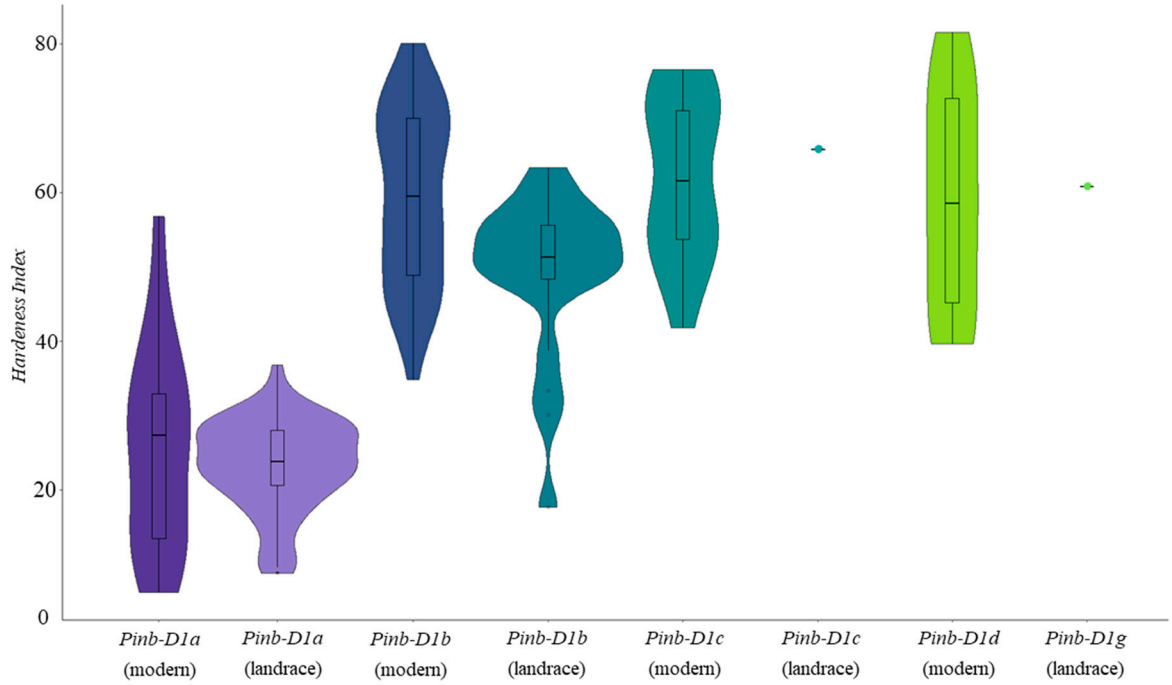

Figure 3. Hardness index ranges for individual *Pinb* alleles divided into modern wheats and landraces. The widths of the figures indicates the frequency of occurrence of a given hardness index.

The tested genotypes were classified, as proposed by Corona et al. [41] into three classes, namely soft, medium hard, and hard types with the SKCS values in the range of 15–40, 41–70, and 71–95 respectively. In the modern cultivars group, 20 genotypes were classified as soft type, 74 as medium hard, and 24 as hard type. In the group of landraces, 61 genotypes were classified as soft and 19 as medium hard.

The protein content in the tested landraces ranged from 13.10% to 23.15%. The starch content ranged from 55.85% to 67.70%, and the wet gluten content ranged from 28.05% to 53.15% (Supplementary Table S3). None of the above values were significantly correlated at $p < 0.05$ with the hardness measured by SKCS. The correlation coefficient between grain hardness and protein, starch and wet gluten content calculated for all landraces was −0.18, 0.19, and −0.16, respectively. The same correlation coefficients calculated for the wild-type *Pinb* landraces were −0.18 (SKCS vs. protein content), 0.15 (SKCS vs. starch content), and −0.03 (SKCS vs. wet gluten content), and for the mutant landraces, these values were −0.41, 0.36, and −0.45, respectively. The normality distribution of hardness values in each allele group was verified by the Shapiro–Wilk test. The test was positive only for the *Pinb-D1b* group in modern wheats. The Kruskal–Wallis test was performed to compare variable distributions between *Pinb* allele groups with a breakdown into modern wheat and landraces, and hardness, and excluding single observations (*Pinb-D1c* and *Pinb-D1g* in landraces). The results proved to be statistically significant (p-value $< 2.2 \times 10^{-16}$). In the next step, the Dunn test was performed in order to establish which groups were different from each other. The SKCS values of modern wheats and landraces with *Pinb-D1b*, *Pinb-D1c*, or *Pinb-D1d* were significantly different compared to genotypes with wild-type *Pinb* allele (Table 2). There were no significant differences between genotypes carrying the *Pinb-D1b*, *Pinb-D1c*, or *Pinb-D1d* alleles (Table 2).

Table 2. Dunn test for multiple comparisons of SKCS hardness in six allele groups. (L)—landraces, (M)—modern cultivars and breeding lines.

Tested Pairs	Z	P.unadj	P.adj	Difference
Pinb-D1a (L)–Pinb-D1b (L)	−4.931	0.000	0.000	True
Pinb-D1a (L)–Pinb-D1b (M)	−9.762	0.000	0.000	True
Pinb-D1a (L)–Pinb-D1c (M)	−6.448	0.000	0.000	True
Pinb-D1a (L)–Pinb-D1d (M)	−6.873	0.000	0.000	True
Pinb-D1a (M)–Pinb-D1a (L)	0.158	0.875	1.000	False
Pinb-D1a (M)–Pinb-D1b (L)	−3.588	0.000	0.005	True
Pinb-D1a (M)–Pinb-D1b (M)	−6.025	0.000	0.000	True
Pinb-D1a (M)–Pinb-D1d (M)	−5.096	0.000	0.000	True
Pinb-D1a (M)–Pinb-D1c (M)	−5.053	0.000	0.000	True
Pinb-D1b (L)–Pinb-D1c (M)	−1.926	0.054	0.812	False
Pinb-D1b (L)–Pinb-D1d (M)	−1.688	0.091	1.000	False
Pinb-D1b (M)–Pinb-D1b (L)	2.042	0.041	0.617	False
Pinb-D1b (M)–Pinb-D1c (M)	−0.516	0.606	1.000	False
Pinb-D1b (M)–Pinb-D1d (M)	−0.058	0.954	1.000	False
Pinb-D1c (M)–Pinb-D1d (M)	0.394	0.694	1.000	False

4. Discussion

We analyzed grain hardness within a collection of various wheat cultivars, breeding lines currently applied in wheat breeding programs and old landraces collected during multiple expeditions between 1960 and 1976 in Poland. Cultivars were tested both at the molecular level (by determining *Pin* gene allelic status) and at the phenotype level (by measuring wheat kernel SKCS hardness index).

Surprisingly, no allelic variability within the *Pina* gene was observed, although mutant alleles of this gene were detected in many other European cultivars [15,24,42]. All tested cultivars, breeding lines, and landraces had the wild-type *Pina-D1a* allele. This allele, originally described by Gautier et al. [5], was proven to be the most common *Pina* allele among wheat genotypes [10,12]. The second *Pina* allele found in European cultivars was *Pina-D1b*, discovered by Giroux and Morris [9] which is extremely rare. Other *Pina* alleles mainly occurred in landraces from China or India [17,18]. The lack of *Pina* variability in the European modern wheat cultivars and breeding lines tested here indicates negative mutant gene selection during the breeding process.

More common allelic variability was observed for the *Pinb* gene. The *Pinb-D1b* allele, detected in 57% of tested modern cultivars and breeding lines, was the most frequent. The remaining *Pinb* alleles were the wild-type *Pinb-D1a* and the mutant *Pinb-D1c* and *Pinb-D1d*. They were represented by a similar variant number (11–16%). Similar distribution of the same *Pinb* alleles was observed previously in European wheat populations [15,24,43]. As for the landraces, the *Pinb* allele distributions were different. The main, wild-type *Pinb-D1a* allele was found in over 70% of tested landraces. The next most frequent was *Pinb-D1b*, detected in 26% of the tested landraces. *Pinb-D1c* was found only in one variety, and none contained the *Pinb-D1d* allele. It was surprising for us to find a landrace with the *Pinb-D1g* allele. To date, this allele has not been observed in European wheat cultivars. It was reported for the first time in historical varieties from North America [16]. We did not find such high variability among *Pina* and *Pinb* alleles, which were observed in hard wheat cultivars originating from other continents. For example, among those originating from Asia, seven various *Pinb* alleles, including four newly described, such as *Pinb-D1ad*, *Pinb-D1ae*, *Pinb-D1af* and *Pinb-D1ag*, have been found [17]. In another study, nine *Pinb* alleles were described [44]. Therefore, our observation confirms that these four alleles found among modern wheats cultivated in Poland dominate in modern European varieties. This is probably caused by the narrow genetic variability of breeding lines as well as being the result of breeding pressure.

The most commonly used methods of determination of grain hardness include the particle size index (PSI), near-infrared reflectance (NIR) with a properly prepared library, and the single kernel characterization system (SKCS). We decided to use SKCS due to the high reliability and accuracy of

this method, and the possibility of analyzing 300 individual grains in a single run. Based on the SKCS value, wheats can be classified into different categories. The first classification was proposed by Morris et al. [16], who distinguished four groups according to the SKCS hardness index: (I) from 0 to 33, (II) from 34 to 46, (III) from 47 to 59, and (IV) above 60. Slightly different categories were proposed by Corona et al. [41] who divided wheat into three main groups: soft (SKCS 15–40), medium hard (41–70), and hard (71–95). The second wheat classification system, used also by us, is the most common. However, in many regions, other classes are used as well, depending on local needs or regulations. For example, Sharma et al. [45] proposed five classes for a more precise classification of soft and hard cultivars from India. They included very soft (SKCS 34 or less), soft (35–54), medium hard (SKCS 55–74), and hard (90 or more).

It was noted that some of the tested cultivars were classified into groups contrary to ourexpectations based on their allelic status. For example, in case of the *Pinb-D1a*, a group of six cultivars and breeding lines had the SKCS indexes ranging from 31 to 56, with a mean value of 46, which was higher than usual for wild-type alleles. In previous reports, cultivars with both wild-type *Pina* and *Pinb* alleles had low grain hardness values ranging from 7 to 40, with a mean value of 27 [41], or from 15 to 29, with a mean value of 24.5 [16]. Moreover, some cultivars, breeding lines, and landraces with *Pinb-D1b* or *Pinb-D1d* mutant alleles, which were expected to have higher grain hardness values, were classified as soft, with a hardness index of 15 to 40. Similarly, one cultivar and one breeding line carrying *Pinb-D1d* were classified as soft.

Particularly interesting is the landrace with the accession number I31930, (*Pinb-D1b*) of which the hardness index is 17.43 (±18.59). Such a low value has never been reported in the case of the *Pinb-D1b* allele. In five breeding lines and seven landraces, the standard deviation of SKCS hardness was above 20, which might suggest that the lines represented a mixture of hard and soft genotypes. This is possible because landraces have been sourced from farmland or local markets and may be contaminated by seeds from other genotypes. In turn, the breeding lines may not yet be genetically homogenous.

Statistical analyses do not show differences in grain hardness between genotypes with the mutant alleles of *Pinb* in both groups. Statistically significant differences occurred only between genotypes carrying the wild-type allele *Pinb-D1a* and genotypes with mutated alleles, which proved that the mutation causing the amino acid sequence change leads to increased grain hardness.

Differences in grain hardness between the same alleles may be caused by other biochemical components of the grain [11,43,46]. Therefore, we also investigated the total protein content, starch, and wet gluten measured by the NIR method. Unfortunately, no correlation between any of these factors was found. This contradicts the results reported by Salmanowicz et al. [47], who found a strong correlation between grain hardness and protein content. It should be noted however, that in the aforementioned work the grain hardness was measured using the NIR and the PSI methods, whereas in the current study the SKCS was applied. As mentioned earlier, the method of measurement influences the results because each method defines the grain hardness in a slightly different way [46,48,49]. The influence of protein content on grain hardness was also reported in other papers [11,50,51]. Moreover grain hardness might be affected by other grain components such as pentosans, primarily arabinoxylans and arabinogalactans [11,52], and gliadin compositions [50], as well as gluten proteins [53]. Other reasons for differences in grain hardness may be the result of differences in genetic background other than variability in *Pin* genes.

Nirmal et al. [34] described significant differences between the expression of *Pin* genes in hard and soft wheat and concluded that higher expression at earlier stages of grain development causes a softer grain, but this does not fully explain entirety of the variability between the genotypes studied. On the other hand, increased expression of *Pin* genes in rice, durum wheat, or in common wheat caused a significant decrease in grain hardness [27,54,55]. The opposed relationship was shown by the silencing of the *Pin* genes, which resulted in increased grain hardness [25,26]. Another known genetic factor, which might affect grain hardness is nucleotide polymorphisms of the transcription activator SPA (storage protein activator) [56]. In addition many QTLs (quantitative trait locus) have been described

that affect the grain hardness. The QTLs related to grain hardness are located on chromosomes 1A, 1B, 2B, 2D, 3B, 4A, 4B, 5AL, 5BL, 5D, 6A, 6BL, 7A, and 7B [57–62]. It has also been proven that grain hardness is affected by environmental factors [49,50,63]. However, in our investigations all cultivars, breeding lines and landraces grew under the same optimal conditions, so we assume that this factor was eliminated. Another factor, which was proven to influence kernel texture is the grain moisture [11,51]. However, in our research, this parameter was almost identical in all tested lines, with the standard deviation values below 1%.

5. Conclusions

In conclusion, no new *Pin* alleles have been discovered however, the *Pinb-D1g* allele, unique to European wheat, has been found among landraces from Poland. In addition, we found many interesting objects whose grain hardness seemed to be determined not only by *Pina* and *Pinb* alleles but also by other genetic or biochemical factors. In further studies, we aim to unravel the genetic background of these inconsistences.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/8/1075/s1, Table S1: Means for kernel hardness, weight, moisture and diameter of modern wheat. Table S2: Means for kernel hardness, weight, moisture and diameter of Polish landraces. Table S3: Means for protein, starch, and wet gluten contents of Polish landraces. Figure S1: Chromatogram fragments of five alleles found in tested wheat genotypes.

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