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Nanopore Amplicon Sequencing Allows Rapid Identification of Glutenin Allelic Variants in a Wheat Collection

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Abstract: Genetic variation in high molecular weight glutenin (HMW-GS) genes is tightly linked with the breadmaking quality of wheat. Hundreds of different alleles have been identified in HMW-GS genes worldwide. Such huge variability makes it difficult to distinguish them using conventional genotyping methods (for example, SDS-PAGE, SNP detection, etc.). Here, we exploited the nanopore amplicon sequencing technique (Amplicon-Seq) to uncover genetic variants distributed along the full-length sequence of six HMW-GSs, including the promoter and protein-coding regions. We analyzed 23 wheat accessions for allelic variants of HMW-GSs using the Amplicon-Seq and SDS-PAGE methods. We obtained sufficient (>50×) target gene coverage by ONT reads in just one hour. Using the obtained data, we identified numerous single nucleotide polymorphisms and InDels in the protein coding and promoter regions. Moreover, Amplicon-Seq allowed for the identification of new alleles (Glu-A1x1-T) of the Glu-1Ax gene that could not be recognized by SDS-PAGE. Collectively, our results showed that Amplicon-Seq is a rapid, multiplexed, and efficient method for high-throughput genotyping of full-length genes in large and complex genomes. This opens new avenues for the assessment of target gene variation to select novel alleles and create unique combinations of desirable traits in plant breeding programs.

Keywords: nanopore; amplicon sequencing; SDS-PAGE; glutenin genes; wheat

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1. Introduction

Genetic variation in key genes involved in shaping desired agronomic traits is crucial for plant breeding programs, including grain quality and breadmaking characteristics [1]. Over the past 25 years, research has revealed a well-defined correlation between distinct allelic variants of high-molecular-weight glutenin subunits (HMW-GS) and breadmaking quality [2,3]. HMW-GSs are a polymorphic family of wheat storage proteins encoded by three loci (Glu-A1, Glu-B1, and Glu-D1), located on the long arms of chromosome one of the A, B, and D subgenomes, respectively [4–6]. Each Glu locus includes two orthologous genes encoding a large molecular weight x-type subunit (Glu-A1x, Glu-B1x, and Glu-D1x) and a lower molecular weight y-type subunit (Glu-A1y, Glu-B1y, and Glu-D1y) [7]. In addition, y-type subunits have a second proline replaced by leucine in the nanopeptide motif of the central domains, whereas x-type subunits have a unique tripeptide repeat motif [8,9]. Payne and Lawrence's 1983 study discovered 3 Glu-A1 alleles, 11 Glu-B1 alleles, and 6 Glu-D1 alleles [10]. During the last 40 years, over 200 alleles for the HMW glutenin loci [11] have been discovered in local wheat varieties, mutants, and wild species [11–14]. The combination of HMW-GSs significantly affected grain and bread quality. The most

valuable x- and y-subunit allele combinations were as follows: Glu-A1a (Glu-A1x), Glu-A1b (Glu-A1x2*), Glu-B1b (Glu-B1x7 and Glu-B1y8), Glu-B1f (Glu-B1x13 and Glu-B1y16), Glu-B1i (Glu-B1x17 and Glu-B1y18), and Glu-D1d (Glu-D1x5 and Glu-D1y10). The Glu-B1al (Glu-B1x7) allele also strengthens dough [8,15,16]. Introducing the Ay21* subunit gene affects both grain protein content and dough quality [17,18].

Traditionally, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has been applied to assess allelic variants of HMW-GSs [13] and is now frequently used in breeding practice [19]. However, some HMW-GSs are quite difficult to distinguish from each other on SDS-PAGE electropherograms because of their similar mobility in the gel. It is particularly difficult to distinguish, for example, subunits 2 and 2*, as well as a combination of subunits 14 + 15 and 20 [20]. The HMW-GS identification problem can be solved using RP-HPLC [21] and MALDI-TOF-MS [3] techniques. However, these techniques are time-consuming, require high-precision equipment, and cannot be scaled to evaluate breeding material. Allelic variants of HMW-GSs are also detected by DNA markers, including KASP markers, enabling the differentiation of high-molecular-weight glutenin subunits, even when they have similar molecular weights [7,22]. For example, KASP markers have been used to accurately detect alleles of the Glu-1 locus by identifying single nucleotide polymorphisms (SNPs) in gene sequences. The relationship between some SNPs and the rheological properties of dough has been demonstrated [23,24]. This technique permits the tracking of most Glu-A1, Glu-B1, and Glu-D1 alleles, even those that are challenging to detect via SDS-PAGE, such as Glu-A1-2 gene alleles encoding the y-type subunit of the A-subgenome. However, this analysis required a set of 17 markers for comprehensive HMW-GS identification, making this procedure time-consuming and complicated. Another method for detecting distinct alleles of HMW-GSs is optimized RP-UPLC, which offers rapid and precise HMW-GS identification. Nevertheless, SDS-PAGE may still be utilized for specific alleles such as Bx7, Bx 17, By 8*, By 9, and By 15. RP-UPLC works solely with protein sequences and does not reveal gene sequences, thus limiting its capacity to assess gene expression levels. Furthermore, it cannot monitor the allelic state of Glu-A1-2, which is frequently unexpressed in cultivated varieties.

Whole-genome sequencing (WGS) of different wheat varieties and available high-quality wheat genome assemblies provide comprehensive insights into genetic variation [25,26]. Nonetheless, WGS is not a rapid and expensive method for assessing the sequence variation of target genes in large wheat genomes. Instead, target DNA amplification followed by Sanger sequencing or next-generation short-read sequencing (NGS) has also been used [25,26]. While Sanger sequencing has been widely used to characterize gluten proteins [24], its application is limited by the maximum template length of 300–1000 bp, which is less than the length of HMW-GS genes (>3Kb) [12,27,28]. In turn, short-read sequencing of PCR products after their fragmentation has limitations in sequencing and assembly of genes with repetitive structures, such as HMW-GS, while this method has high throughput and a low error rate. In our previous study, we employed novel Cas9-mediated target nanopore sequencing (nCATS, [29]) to sequence multiple glutenin genes (specifically Glu-A1x, Glu-B1x, and Glu-B1y) and their respective promoters in hexaploid triticale [30]. This method is particularly useful for investigating gene variability and DNA methylation profiles [30]. However, this method is not high-throughput, and the genes of only one variety have been sequenced at once, resulting in relatively low gene coverage by nanopore reads. An alternative method for target gene sequencing is the PCR amplification of genes, followed by fragmentation and short-read sequencing [24]. Therefore, alternative approaches are required for high-throughput sequencing of the entire HMW-GS gene to identify novel and potentially valuable alleles.

Long-read Nanopore sequencing of amplicons (ONT Amplicon-Seq) has recently been used in several studies to study microbial diversity [31]. For example, ONT Amplicon-Seq was used to sequence 4.4 Kb ribosomal RNA operons from a microbial community [32] to decrease the error rate of ONT Amplicon-Seq reads (usually around 5–25%) [33], and unique molecular identifiers (UMIs) were used [32]. The UMI application resulted in a

dramatic increase in sequence accuracy of up to ~98%. Errors in individual ONT reads are randomly distributed. This distribution allows for the identification of SNPs without UMIs, even at medium-sequence coverage [30]. Only a few reports have used ONT Amplicon-Seq to elucidate the genetic variation in eukaryotic genes [34–36]. For example, Nanopore Amplicon-Seq has been used to validate IGVH mutations in clinical studies of FFPE (Nz66) gliomas. While this study amplified relatively small PCR products within the 300–600 bp size range, these products proved adequate for obtaining reliable clinical validation data, demonstrating the feasibility of this approach for detecting single nucleotide variants [36]. ONT Amplicon-Seq has been performed to investigate the effects of plasmotype on chlorophyll fluorescence in barley (*Hordeum vulgare* ssp. spontaneum) [37]. Thus, a systematic assessment of ONT Amplicon-Seq for the survey of allelic variation of multiple target genes in crops has not yet been conducted.

Here, we tested the Amplicon-Seq method to uncover allelic variation in six HMW-GS genes in a wheat collection of 23 varieties and compared the results with SDS-PAGE. Both approaches have identified various alleles of Glu genes in a collection of wheat accessions. Nanopore amplicon sequencing provided deep coverage (>50×) and allowed the uncovering of SNPs and InDels in Glu genes after just one hour of sequencing. Moreover, ONT Amplicon-Seq allowed us to detect novel allelic variants that could not be identified by SDS-PAGE. Additionally, Nanopore amplicon sequencing detected differences both in the coding region of glutenin genes and in the promoter regions, which cannot be performed using SDS-PAGE electrophoresis. In summary, our study showed that nanopore amplicon sequencing is a rapid and multiplex approach for the analysis of genetic variation of full-length genes in plant species with large and complex genomes. This paves the way for the development of full-length target gene panels that can be applied in plant breeding processes when the number of alleles in a gene is high.

2. Materials and Methods

2.1. Plant Material

A total of 23 cultivars of spring bread wheat cultivars were used in this study. Nineteen samples of hybrid origin were obtained from the International Maize and Wheat Improvement Center (CIMMYT), and four samples were of different geographical origins (Supplementary Table S1). The bread wheat line Chinese Spring (CS) was used as a standard for HMW-GS by SDS-PAGE. Grains of each cultivar were separated into two parts, embryo and endosperm, to investigate one grain by amplicon sequencing and SDS-PAGE.

2.2. SDS-PAGE of HMW-GS

Proteins were extracted from individual half-grains using the sequential procedure described by Singh et al. [38]. Three grains were analyzed per variety. Electrophoresis of HMW-GSs was performed on a vertical gel (200 × 183 × 1 mm) (Bio-Rad Protean XL apparatus, Hercules, CA, USA) according to the SDS-PAGE protocol described by Barnlard et al. [39], with modifications: Time of running was 20 h with 20 mA per gel. The HMW-GSs were identified by their electrophoretic mobility relative to the subunits of the Chinese Spring variety, which had a composition of AxN/Bx7+By8/Dx2+Dy12. The nomenclature of Payne and Lawrence [10] was used for HMW-GS.

2.3. DNA Isolation

Total DNA was extracted from the embryo seedlings using the CTAB protocol [40], which were germinated in the dark at room temperature on wet filter paper disks. Five-day-old seedlings were homogenized in liquid nitrogen. It takes 500 mg of plant material for extraction using 500 µL CTAB1. Process of DNA isolation was carried out according to the published protocol (https://www.protocols.io/view/plant-dna-extraction-and-preparation-for389-ont-seque-bcvyiw7w, accessed on 4 September 2021). The DNA pellet was washed with 70% ethanol and resuspended in 50 µL of nuclease-free water. DNA concentration and integrity were estimated using Nanodrop (Nanodrop Technologies,

Wilmington, CA, USA) and gel electrophoresis using a 1% agarose gel with ethidium bromide staining.

2.4. PCR Amplification of Glutenin Subunits

Primer pairs were designed to amplify promoter and coding regions. The amplicon lengths ranged from 2600 bp (Glu-A1x) to 5000 bp (Glu-D1y) (Supplementary Table S2). Primer pairs were used to detect the longest conserved region of each gene. All primers were designed using Primer 3.0 software (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (accessed on 1 September 2022) and checked by PrimerBLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/, accessed on 22 November 2023).

To optimize PCR conditions, we employed a mix of PCR enzymes, combining proofreading and highly processive types, along with hot-start antibodies. These antibodies effectively suppressed polymerase activity at room temperature, thus preventing non-specific amplification and enabling a versatile reaction setup. To successfully amplify Glu-B1x and Glu-B1y, PCR was performed using Encyclo DNA polymerase (Evrogen, Moscow, Russia) and magnesium-free encyclo buffer. $MgCl_2$ and DMSO concentrations were used for PCR optimization to achieve the desired results. $MgCl_2$ (1 mM) influences polymerase activity, primer-template interactions, and the overall specificity and efficiency of DNA amplification. DMSO (3%) was used in PCR to hinder the formation of secondary structures in either the DNA template or DNA primers. We conducted long-range PCR to amplify four fragments (Glu-A1x, Glu-A1y, Glu-D1x, and Glu-D1y) using Biolabmix LR HS polymerase and 5% DMSO according to the manufacturer's instructions (Biolabmix, Novosibirsk, Russia). The PCR conditions were specific for each subunit (Supplementary Table S3). The obtained PCR results were visualized via gel electrophoresis using a 1% agarose gel with ethidium bromide staining.

After amplification, 4 kb full-length products of Glu-B1x were purified from a 1% agarose gel using a Cleanup Standard kit (Evrogen, Moscow, Russia) according to the manufacturer's instructions. PCR products of other glutenin subunits were equalized in concentration and pooled into one sample according to the cultivar. Pooling amplicons were purified using 1.8× Agencourt AMPure XP Beads (Beckman Coulter, Pasadena, CA, USA) in accordance with the manufacturer's instructions. Purified amplicons of Glu-B1x and pooled amplicons were equalized in concentration and pooled in one final sample according to the cultivars. The pooled amplicon concentration and integrity were estimated using Nanodrop (Nanodrop Technologies, Wilmington, CA, USA) and Qubit (Qubit ds-DNA BR Assay Kits, Thermo Fisher Scientific, Waltham, MA, USA) and checked by gel electrophoresis.

2.5. Library Preparation and Nanopore Sequencing

In this study, we did not use phosphorylated primers for PCR amplification. In this case, the 5′-ends of the amplicon are non-phosphorylated and need to be treated by a T4 polynucleotide kinase to introduce 5′-phosphate. The phosphorylation mix was prepared in 22 µL with the use of 10 µL pooling amplicons (200 ng), 2.2 µL 10× T4 polynucleotide kinase reaction buffer, 2 µL dATP (1 mM), 0.2 µL T4 polynucleotide kinase (2 units), and nuclease-free water up to the necessary volume. Incubation was performed on a thermocycler with the following program: 37 °C for 30 min, and then 65 °C for 20 min. Phosphorylated amplicons were purified using an equal volume of Agencourt AMPure XP Beads (Beckman Coulter, Pasadena, CA, USA), and 100 µL of freshly prepared 70% ethanol was washed twice. The obtained amplicons were resuspended in 4.5 µL nuclease-free water in a new LoBind tube for downstream analysis.

For Nanopore sequencing, a library was prepared from 23 pooled samples using the nanopore native barcoding genomic DNA SQK-NBD110-24 (Oxford Nanopore Technologies, Oxford, UK), with some modifications in the process of using the NEBNext Companion Module for Oxford Nanopore Technologies Ligation Sequencing (New England Biolabs, MA, USA). Briefly, ~100 ng of each pooling sample in 4.5 µL was mixed with 0.5 µL Native

Barcode and 5 µL Blunt/TA Ligase Master Mix and incubated on a Hula mixer for 10 min at room temperature. Purification by Agencourt AMPure XP Beads was performed during phosphorylation. Each of the 12 barcoded samples was resuspended in 2.7 µL of nuclease-free water and transferred to a new LoBind tube for adapter ligation. Then, ~32.5 µL pooled and barcoded amplicons were mixed with 10 µL NEBNext Quick Ligation Reaction Buffer (5X), 5 µL Quick T4 DNA Ligase, and 2.5 µL Adapter Mix II (AMII). Adapter Ligation Mix was incubated on a Hula mixer for 10 min at room temperature. Double washing was performed using 125 µL of Short Fragment Buffer (SFB). Incubation was performed in a water bath at 37 °C for 10 min, and then for 5 min at room temperature. Sequencing was carried out using MinION and a flow cell SQK-LSK109. Basecalling was performed by Guppy (Version 6.3.8). The obtained reads were aligned to the reference sequences of HMW-GS genes from NCBI (Glu-1A: AF145590.1, KJ531446.1, M22208.2, MF568383.1, EU984510.1; Glu-1B: DQ119142.1, FM955452.1, MH108092.1, KC254854.1, JN255519.1, X61026.1, EU137874.1, EF540765.1, KF430649.1; Glu-1D: BK006460.1, AB485591.1, X12929.2, JF736016.1) using minimap2 [41]. The obtained BAM files were visualized using JBrowse2 [42].

2.6. PCR Validation of the Deletion in Glu-1Bx7 Gene

To confirm the deletion in the promoter region of Glu-1Bx7 (approximately 60 bp), as identified by Nanopore amplicon sequencing, we used the primers provided in Supplementary Table S4. PCR was conducted using Encyclo DNA polymerase (Evrogen, Moscow, Russia) following the manufacturer's instructions. The PCR conditions were 95 °C for 5 min; 33 cycles of 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 35 s; and final elongation at 72 °C for 3 min.

3. Results

3.1. Assesment of HMW-GS Alleles in Different Wheat Varieties Using SDS-PAGE

To evaluate the allelic variants of high molecular weight glutenin (HMW-GS) present in the accessions of our wheat collection, individual wheat grains were analyzed using SDS-PAGE and ONT Amplicon-Seq. Each grain of the 23 selected wheat accessions was divided into two parts (Figure 1).

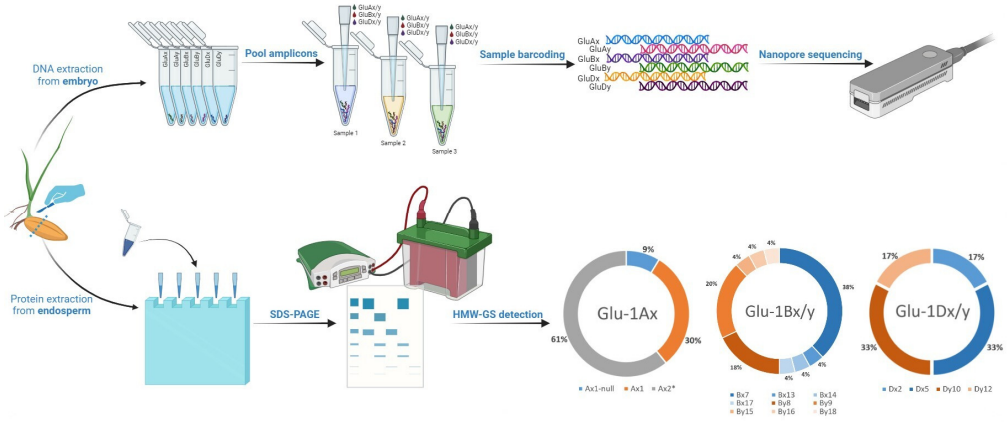

Figure 1. Schematic depicting the identification of HMW-GSs by ONT Amplicon-Seq and SDS-PAGE. Each grain was divided into two parts: the endosperm was used for SDS-PAGE, and seedlings obtained from the embryo were subjected to DNA isolation, PCR amplification with specific primers, and barcoding. Barcoded amplicons from different grains were mixed and sequenced on a single MinION flow cell. The percentage of wheat accessions carrying different HMW-GSs as assessed by SDS-PAGE is shown at the bottom. The figures were generated using BioRender (https://biorender.com/, accessed on 3 June 2023).

SDS-PAGE analysis of the allelic variants of HMW-GSs showed (Supplementary Table S5) that seven accessions carried the Glu-A1x1 allele, 14 accessions carried the Glu-A1x2 * allele, and 2 accessions carried the Glu-A1x-null allele. The studied accessions had the following

combinations of allelic variants of the Glu-B1 locus: Glu-B1x7 and Glu-B1y8—8 accessions, Glu-B1x7 and Glu-B1y9—9 accessions, Glu-B1x13 and Glu-B1y16—2 accessions, Glu-B1x14 and Glu-B1y15—2 accessions, and Glu-B1x17 and Glu-B1y18—2 accessions. The lowest allelic diversity was found at the Glu-D1 locus: eight accessions carried Glu-D1x2 and Glu-D1y12; 15 accessions carried Glu-D1x5 and Glu-D1y10 (Figure 2).

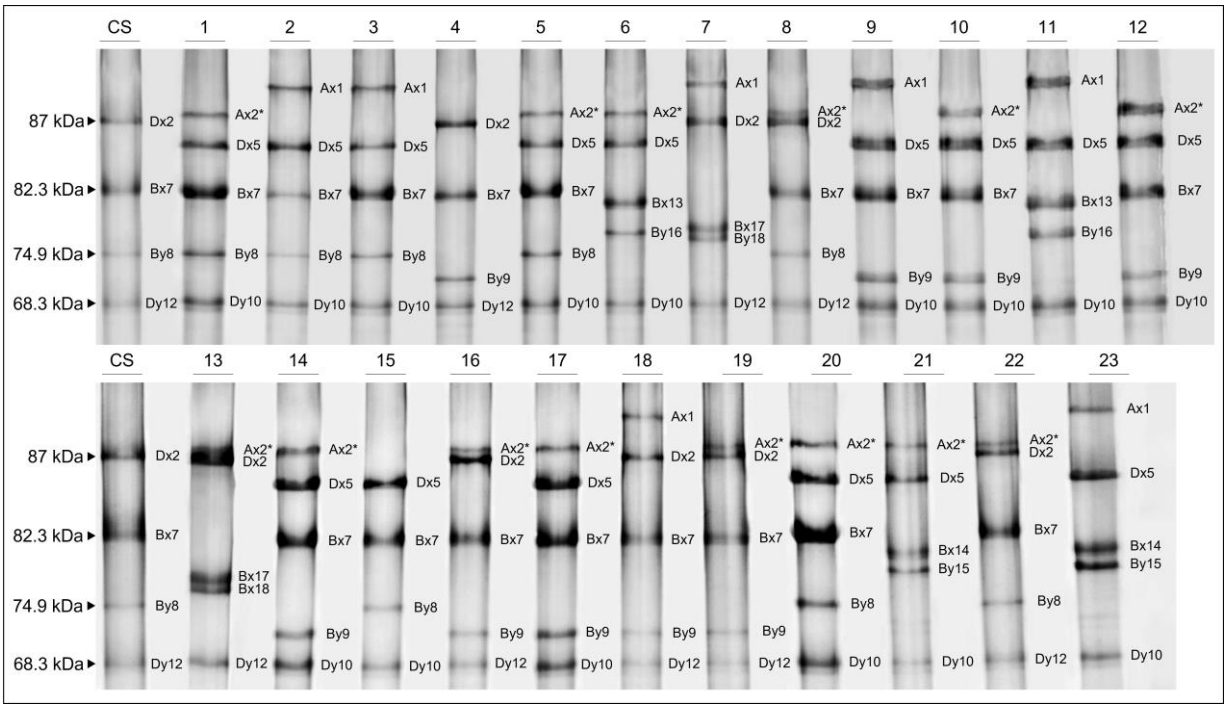

Figure 2. SDS-PAGE separation of the high-molecular-weight glutenin subunits found in the studied bread wheat accessions: 1. MC № 23; 2. MC № 35; 3. MC № 59; 4. MC № 65; 5. MC № 66; 6. MC № 67; 7. MC № 70; 8. MC № 79; 9. MC № 147; 10. MC № 150; 11. MC № 151. 12. MC № 152. MC mean "Mexican Collection", 13. MC № 153; 14. MC № 178; 15. MC № 187; 16. MC № 215; 17. MC № 217; 18. MC № 220; 19. MC № 223; 20. Glenlea; 21. Bombona; 22. Agatha; 23. Kanyuk. MC means "Mexican Collection", CS—Chinese Spring.

3.2. Preparation of PCR amplicons of HMW-GS genes

Next, we performed Amplicon-Seq of full-length HMW-GS genes. For this, we designed custom primers to obtain PCR products covering the promoter (−1500 bp from the transcription start site) and coding region of the six HMW-GS wheat genes. The primers were located in regions that were conserved between different alleles of the HMW-GS genes, as determined by BLAST analysis. PCR conditions were optimized for each primer pair. The obtained PCR products had the following length: 4.3 kb and 2.6 kb for Glu-A1x and Glu-A1y, 3.9 kb and 3.6 kb for Glu-1Bx and Glu-1By, and 3.0 kb and 5.0 kb for Glu-D1x and Glu-D1y, respectively (Figure 3).

In total, 138 PCR products (Figure 3) were purified and mixed to obtain the final samples for barcoding (Figure 1). Notably, because we used conventional non-phosphorylated primers, the obtained PCR products were phosphorylated, barcoded, and sequenced using a single MinION flow cell. The analysis of read generation during the sequencing procedure showed that a read coverage of 50×–150× was obtained for each amplicon of an individual wheat accession after just one hour of sequencing. In total, we obtained approximately 215,000 reads with an N50 value of ~3.2 kb. On average, approximately 9000 reads were associated with each barcode. The results demonstrate that ONT Amplicon-Seq can rapidly generate a sufficient amount of data in a short time.

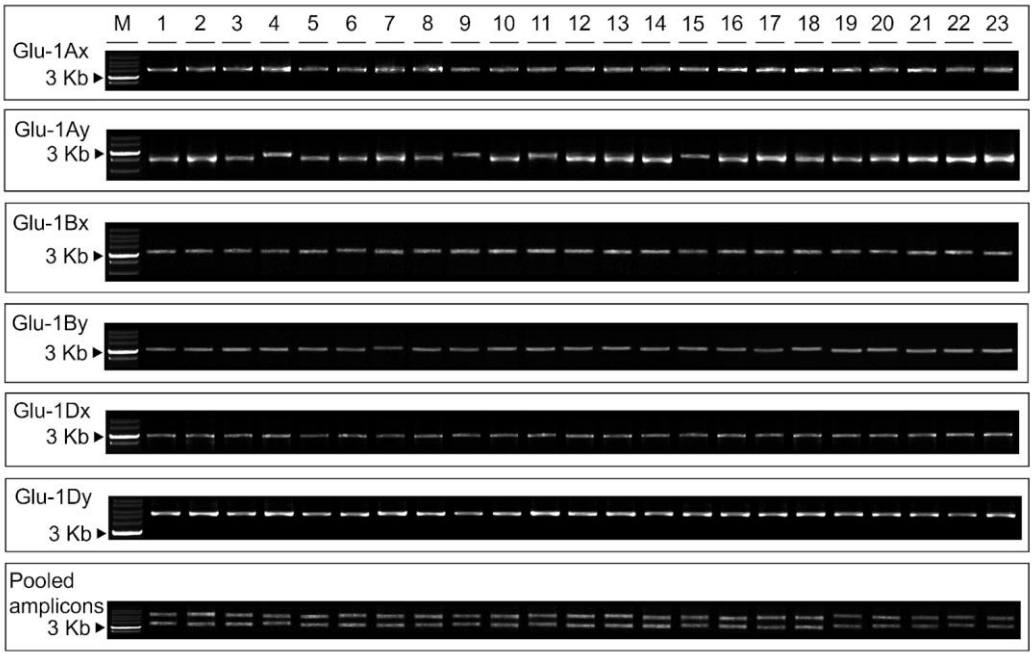

Figure 3. Gel electrophoresis of the PCR products after amplification of the target regions (promoter + coding sequence) of the six glutenin genes (Glu-A1x, Glu-A1y, Glu-B1x, Glu-B1y, Glu-D1x, Glu-D1y) and the mixes of purified and pooled amplicons for 23 bread wheat accessions. Individual wheat genotypes were (see material and methods for details): MC №23, MC №35, MC №59, MC №65, MC №66, MC №67, MC №70, MC №79, MC №147, MC №150, MC №151, MC №152, MC №153, MC №178, MC №187, MC №215, MC №217, MC №220, MC №223, Glenlea, Bombona, Agatha, Kanyuk. The DNA marker Sky-High (Biolabmix, Novosibirsk, Russia) (M) MC means "Mexican Collection".

3.3. Analysis of HMW-GS Allele Sequences Deduced by ONT Amplicon-Seq

To identify the allelic variants of each of the six HMW-GSs, the obtained ONT reads were aligned to the reference set of the HMW-GSs. Then, the SNPs and InDels were detected, and the corresponding known HMW-GS allele ids were assigned based on their identity to the sequences from NCBI.

The analysis of ONT data for Glu-A1x variation across 23 wheat samples revealed that 14 (61%) samples had the Glu-A1x2* allele, two accessions (9%) had the Glu-A1x-null allele, and seven accessions (30%) had the Glu-A1x1 allele, corroborating the SDS-PAGE data. The sequence of Glu-A1x genes of two wheat accessions (MC №147, MC №151) had a specific SNP (3136C -> T) in the coding region of the gene, distinguishing them from the known sequences of the Glu-A1x1 allele (Figure 4). This substitution is non-synonymous, causing the replacement of Pro by Ser. SDS-PAGE identified the Glu-A1x allele as Glu-A1x1. The new Glu-A1x allele was called Glu-A1x1-T.

Glu-A1y is typically inactive in cultivated hexaploid wheat varieties, making SDS-PAGE analysis impossible. ONT Amplicon-Seq enabled analysis of the Glu-A1y sequence. We found two Glu-A1y alleles in the wheat collection. These alleles were identical to two known alleles deposited at NCBI: Glu-A1y-d (MF568383.1 [43]) and A1y/Td-s (EU984510.1, Glu-A1-2 [26]). The Glu-A1y-d allele was detected in 16 accessions (70%) in our wheat collection. The remaining seven accessions (30%) carried the inactive Glu-A1-2 allele [26].

Amplicon-Seq ONT read mapping to the known Glu-B1x alleles revealed the presence of a diverse panel of Bx alleles in our wheat collection: Bx7 (17 accessions, 73%), Bx17 (2 accessions, 9%), Bx13 (2 accessions, 9%), and Bx14 (2 accessions, 9%). The results of ONT Amplicon-Seq and SDS-PAGE were concordant. Using ONT data, we easily uncovered a ~60 bp deletion and SNP (C -> G) present in the promoter of the 1Bx7 allele (Figure 5). To confirm these findings, we developed unique primer pairs and performed PCR. The

PCR results and Sanger sequencing verified the presence of the deletion in the Glu-B1x7 promoter in the 12 wheat accessions (Supplementary Table S5).

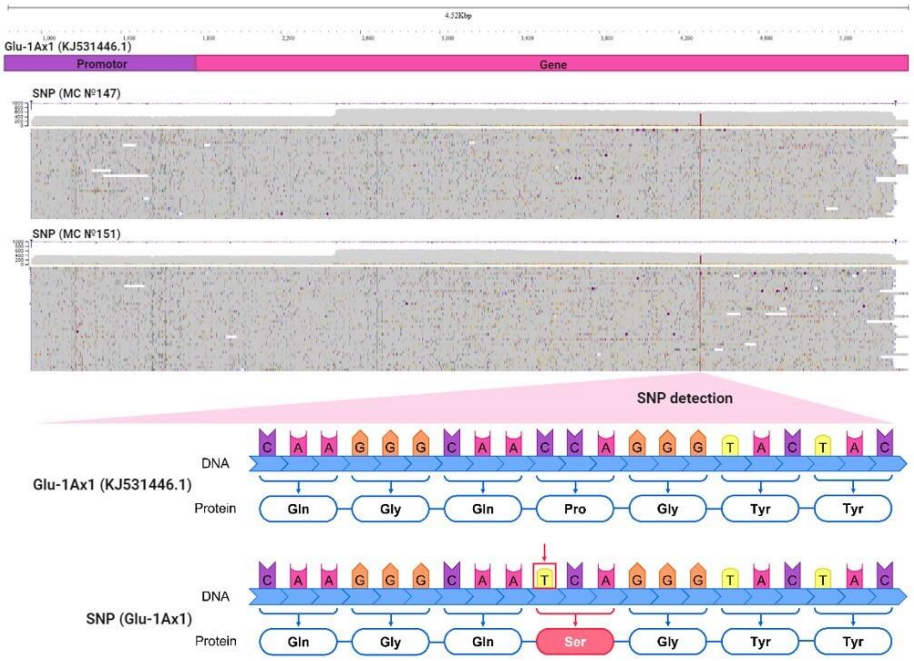

Figure 4. New allele (Glu-A1x1-T) of the Glu-A1x gene. JBrowse2 snapshot of ONT Amplicon-Seq read alignment to the Glu-B1x1 reference allele. A non-synonymous substitution identified within the coding region of the Glu-A1x1 gene is shown.

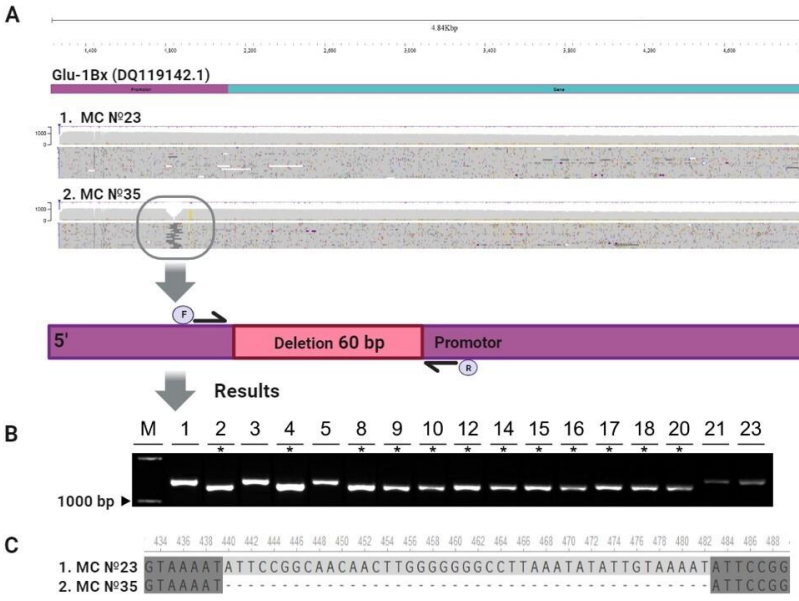

Figure 5. Sequence analysis of Glu-1Bx (**A**) Read alignment of the two accessions to the reference sequence of Glu-1Bx and the positions of primers for InDel validation. (**B**) PCR results with specific primer pairs flanking the InDels of Glu-1Bx7 variants. Individual wheat genotypes were as follows: 2. MC № 35, 4. MC № 65, 8. MC № 79, 9. MC № 147, 10. MC № 150, 12. MC № 152, 14. MC № 178, 15. MC № 187, 16. MC № 215, 17. MC № 217, 18. MC № 220, 20. MC № 223 (with 60 bp deletion); 1. MC № 23, 3. MC № 59, 5. MC № 66, 21. Glenlea, 23. Agatha (without deletion). (**C**) Identification of deletion after multiple alignment of sequences obtained by Sanger sequencing. DNA marker Step50 plus (Biolabmix, Novosibirsk, Russia) (M). Alleles with deletion are indicated by star (*). MC mean "Mexican Collection".

Analysis of Glu-B1y sequences by ONT Amplicon-Seq revealed that MC №151 and MC №67 samples carried alleles similar to the known By16 and By19* alleles, respectively. The two alleles differed by 27 bp in-frame InDel. However, SDS-PAGE indicated the presence of the By16 allele in all analyzed wheat accessions suggesting 9aa deletion is too small to be detected by SDS-PAGE. Analysis of the other 21 wheat samples revealed that 8 (35%) accessions had the Glu-B1y8 allele, and 9 accessions (40%) had Glu-B1y9, Glu-B1y15 (9%), and Glu-B1y8 (9%) alleles by two accessions, corroborating with the SDS-PAGE data.

For Glu-1D genes, the alleles identified by SDS-PAGE correlated with those identified by sequencing. Both methods revealed the following distribution of alleles: 8 accessions (35%) had Glu-D1x2 and Glu-D1y12 alleles, and 15 accessions (65%) had Glu-D1x5 and Glu-D1y10 alleles.

Thus, the results showed that ONT Amplicon-Seq can identify all alleles of HMW-GS genes and, in some cases, has higher precision than SDS-PAGE. Moreover, using ONT data, we found the previously unknown allele Glu-A1x1-T, paving the way for further functional characterization.

4. Discussion

In this study, we successfully applied the ONT Amplicon-Seq technique to rapidly determine allelic variants of six full-length HMW-GS in a collection of 23 wheat accessions of different origins. Fourteen alleles were identified and verified using Sanger sequencing and SDS-PAGE. Many of these alleles have been previously observed in cultivated wheat varieties and in genetic collections. Ten alleles (Glu-A1x1, Glu-A1x2*, Glu-B1x7 and Glu-B1y8, Glu-B1x13 and Glu-B1y16, Glu-B1x17 and Glu-B1y18, Glu-D1x5 and Glu-D1y10) are considered advantageous for baking and are commonly used in breeding programs worldwide [8,15].

We demonstrated that the ONT Amplicon-Seq and SDS-PAGE methods have their own advantages and disadvantages (Table 1). Although SDS-PAGE is a straightforward and accessible method for assessing the baking potential of wheat breeding material through HMW-GS identification, it has distinct limitations including low processing capacity.

Table 1. Characteristics of SDS-PAGE and Nanopore Amplicon-Seq for HMW-GSs identification.

Characteristics	SDS-PAGE	Nanopore Amplicon-Seq
Target	Protein	Nucleic acid
Analyzed material	Endosperm	DNA containing parts
Ability to analyze an individual plant	+	+
Number of simultaneously tested samples	Up to 40	Up to 96
Operating time of the device (excluding sample preparation and subsequent detection)	Up to 20 h	About 1 h to achieve the required coverage
The number of simultaneously studied HMW-GS loci	Five	Multiple
Analysis range	5–250 kDa	250–10,000 bp
Possible cause of the error	Errors of low gel resolution and the similar electrophoretic mobility of some subunits	PCR and sequencing errors
Reliability of identification	+	+++
Search for non-annotated alleles	±	+++
Portability	–	+++

Both traditional SDS-PAGE and Nanopore Amplicon-Seq offer the ability to analyze individual plants, which is particularly valuable for assessing breeding material during early hybrid generations. Amplicon-Seq DNA from the leaf material of mature plants can be used for ONT. SDS-PAGE, on the other hand, requires at least half a grain without a germ, raising concerns about the further viability of the remaining half with the germ.

SDS-PAGE is well known for its relative simplicity and affordability of reagents and equipment. However, SDS-PAGE characterizes HMW-GSs based on their relative mobility in the gel, which does not always correspond to their actual molecular weights. In contrast, ONT Amplicon-Seq results in the sequencing of an entire target locus, providing information on gene and promoter nucleotide sequences. ONT Amplicon-Seq can detect different variations, ranging from SNPs to in-frame InDels. This enables the precise identification of known alleles and the discovery of new alleles, characterized by mutations in the coding and promoter regions. The promoter regions of wheat glutenin genes are of particular interest because of their significant variability and direct impact on gene expression and, consequently, the quality of wheat flour [44]. Such novel alleles can potentially explain cases where a high Glu-score does not translate to good bread quality, or vice versa [8]. For example, some researchers associate the high bread quality of wheat varieties from Russia [45], Turkey [46], and India [47] with the Glu-D1a allele and recently discovered alleles, such as Dy12.7 [27] and 1Dy12** [14], which have the same electrophoretic mobility as Dy12 but determine baking quality as Dy10.

Another important characteristic is the time required for allele identification (Figure 6). The results demonstrate that ONT Amplicon-Seq significantly shortens the analysis time compared to SDS-PAGE. Various sources indicate the duration of electrophoresis from 2.5 to 17 h [3,38] without considering sample preparation and visualization of protein profiles. However, the task of optimizing SDS-PAGE parameters is intricate and time-consuming, involving numerous adjustments, including pH level, sample size, ammonium concentration in terms of sulfate (APS), and staining duration, to obtain a highly accurate image for the registration of the most polymorphic proteins, with molecular weights ranging from 80–130 kDa. Protein separation on the polyacrylamide gel took 20 h. The final stage of protein analysis, involving fixation, staining, washing, and subsequent detection of the obtained subunits, is typically the least time-consuming, requiring approximately 1 h. In contrast, ONT Amplicon-Seq requires significantly less time for sample preparation and sequencing, while the data analysis step is more time-consuming than SDS-PAGE.

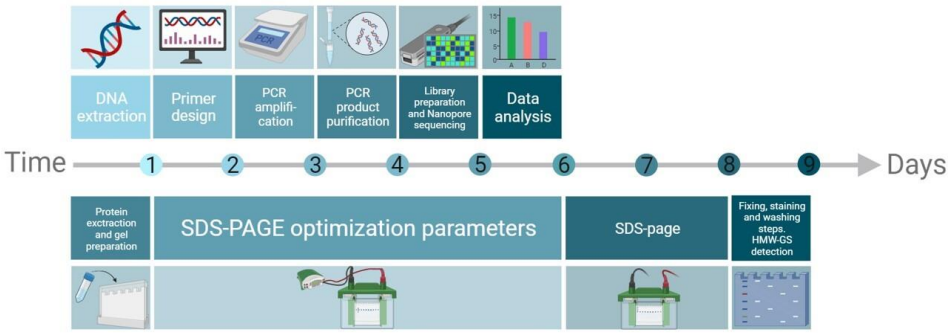

Figure 6. Comparison of timelines for the identification of HMW-GSs using ONT Amplicon-Seq and SDS-PAGE. The top line shows the preparation of amplicons and nanopore sequencing. The bottom line shows protein extraction, optimization of SDS-PAGE, and processing of the obtained results. Each step on the timeline represents one day of time spent. The figures were generated using BioRender (https://biorender.com/, accessed on 3 June 2023).

ONT Amplicon-Seq has several limitations that should be considered. First, the error rate of the ONT reads is still quite high [48]. Polymerase errors during amplification are another source of sequencing errors [32]. Therefore, individual reads cannot be used to determine polymorphisms. To overcome this limitation, sufficient coverage (>30× in

our experience) of the target loci by ONT reads is required, and high-precision DNA polymerase should be used for target amplification. In addition, some DNA conformation forms (e.g., non-B DNA structures) and sequence motifs (e.g., polypurine/polypyrimidine tracts) are more prone to introduce errors during sequencing [49]. Secondly, primer design and PCR optimization are the most time-consuming and critical steps in ONT Amplicon-Seq. Notably, the designed primers should be extensively tested to limit non-specific primer annealing. Additionally, the primer sites should be placed in regions that are well-conserved between different cultivars to ensure uniform amplification of the target loci across genotypes. Third, we performed PCR amplification of individual genes, followed by pooling the PCR products from a cultivar into one tube. This strategy can be challenging when the number of targets is large. In this case, multiplex amplification can be applied, which requires extensive optimization. The number of genes involved in crucial pathways for plant breeding (e.g., grain development [50]) is rapidly growing. Multiplex ONT Amplicon-Seq can be a useful strategy for building ONT-based platforms for simultaneous high-throughput genotyping of multiple genes in plant collections. ONT Amplicon-Seq platforms for full-length genotyping are easily scalable, and novel target genes can be rapidly added. Finally, DNA polymorphisms established by sequencing are not always informative for predicting their effect on protein functions. In the case of novel HMW-GSs, SDS-PAGE and ONT Amplicon-Seq can be complementary approaches to better characterize the consequences of mutations at the protein level.

5. Conclusions

Our results show that the ONT Amplicon-Seq technique is an accurate and rapid method for multiplexed genotyping of full-length target genes in plant species with large and complex genomes. Using ONT Amplicon-Seq, different variations ranging from SNPs to in-frame indels can be easily detected in a short time. This enables the precise identification of known alleles and the discovery of new alleles characterized by mutations in coding and promoter regions. The obtained results provide useful information for the selection of a desirable combination of HMW-GSs for wheat breeding.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14010013/s1, Table S1: 23 cultivars of spring bread wheat were used in this study; Table S2: Primers used for PCR; Table S3: PCR conditions for amplification of glutenin subunits; Table S4: Primers used for SV identifications; Table S5: The HMW glutenin subunit composition of bread wheat cultivars.

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