

## Article

# Development of Genome-Wide Unique Indel Markers for a Heat-Sensitive Genotype in Wheat (*Triticum aestivum* L.)

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**Abstract:** A chromosome segment substituted line (CSSL) represents an ideal resource for studying quantitative traits like thermotolerance. To develop wheat inter-varietal CSSLs with E6015-3S (a heat-sensitive genotype) being the recipient parent, genome-wide unique DNA markers are urgently needed for marker-assisted selection. In this study, 11,016 primer pairs targeting 5036 indel sites were successfully designed for E6015-3S, with an average density of 0.36 indels per Mbp. These primer pairs are believed to be unique and polymorphic in the wheat genome; as gathered from the evidence, (i) 76.18 to 99.34% of the 11,016 primer pairs yielded a single hit during sequence alignment with 18 sequenced genomes, (ii) 83.59 to 90.98% of 1042 synthesized primer pairs amplified a single band in 16 wheat accessions, and (iii) 59.69 to 99.81% of the tested 1042 primer pairs were polymorphic between E6015-3S and 15 individual wheat accessions. These primer pairs are also anticipated with excellent resolvability on agarose or polyacrylamide gels, since most of them have indel sizes from 15 to 46 bp, amplicon sizes from 141 to 250 bp, and polymorphism ratios from 6.0 to 25.0%. Collectively, these primer pairs are ideal DNA markers for inter-varietal CSSL development and more broad applications, like germplasm classification, seed purity testing, genetic linkage mapping, and marker-assisted breeding in wheat, owing to their uniqueness, polymorphism, and easy-to-use characteristics.

**Keywords:** wheat; thermotolerance; inter-varietal CSSL; E6015-3S; indel marker; genome-wide uniqueness



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## 1. Introduction

Global crop productivity is being challenged by the detrimental effects of climate change [1]. Heat stress (HS) is considered more dangerous than other stresses because it significantly affects various plant organs at multiple lifecycle stages [2]. Wheat, a major source of calories and proteins worldwide, is quite sensitive to HS, especially during its reproductive stage. HS can disturb its reproductive physiology and therefore cause a significant decrease in yield [3]. Around one-half of the global wheat area is routinely impacted by heat stress, especially those developing regions with limited access to technology in Asia and Africa [4,5]. Models highlight the risks of crop failures due to rising temperature, with each 1 °C increase in global mean temperature being predicted to decrease the global wheat yield by 6% on average [6].

Advances in genetics and genomics have facilitated the identification of numerous quantitative trait loci (QTLs) influencing HS tolerance in wheat [7]. However, the majority of these QTLs are identified from primary mapping populations and have inadequate genetic effects or mapping resolutions and are therefore difficult to use. Currently, only a few major QTLs conferring HS tolerance have been finely mapped to a sub-megabase level in wheat, i.e., *TaHST1* [8], *qDHY.3BL* [9], and *TaHST2* [10], because heat response phenotypes are difficult to score, varying among growing seasons and requiring substantial inputting time and labor. Further efforts are consequentially needed to better understand the genetic basis controlling wheat HS tolerance.

A chromosome segment substitution line (CSSL) is a type of genetic resource representing a single or a few chromosomal segments from the donor parent in the recurrent parent background [11]. CSSLs that carry a single donor segment are called single-segment substitution lines [12] or near-isogenic lines [13]. Secondary mapping populations like CSSLs eliminate interferences from other QTLs and provide QTL visualization as a single Mendelian factor, and hence they can improve the mapping accuracy of causal genes [14,15]. Accordingly, the utility of CSSLs in the identification of novel QTLs/genes influencing a wide range of traits has been demonstrated in food and commercial crops [11]. Usually, CSSLs are developed by advanced backcrosses and continuous marker-assisted selection (MAS) to keep the amount of donor segments as low as possible. To achieve this, a set of DNA markers, such as CAPS [16], SSRs [17], indels [14], and SNPs [18], is needed to genotype the whole genome.

Wheat CSSLs are mainly developed with an ancestral progenitor being the donor parent in the background of a cultivated variety so as to incorporate possible novel alleles for yield [19], quality [20], and resistance [21,22]. Among studies like these, genome-specific markers targeting an individual alien chromosome were frequently used in screening wheat-alien introgression lines [23]. Most of the available inter-varietal chromosome substitution lines (CSLs) in wheat were developed decades ago before the advent of DNA markers, a laborious and time-consuming process that requires the prior creation of monosomic series [24]. To the best of our knowledge, the systematic construction of inter-varietal CSSLs in regard to wheat has been infrequently studied or reported.

To facilitate the efficiency of MAS during inter-varietal CSSL construction, a set of PCR-based DNA markers with ideal coverage and uniqueness in the wheat genome is needed. However, no such set of DNA markers is currently available in wheat. Even the most commonly used SSRs cannot meet this demand due to their multi-locus and redundancy characteristics [25]. In addition, accessibility to SSRs is not easy due to the fact that intensive labors and complex procedures are required for polyacrylamide gel separation. Indel markers provide an alternative for agarose gel electrophoresis, which is easy to operate, when the size of insertion/deletion is relatively large. The goal of our ongoing research is trying to solve this problem by developing a set of indel markers for a heat-sensitive genotype (E6015-3S) [8] with special emphasis on their genome-wide uniqueness and easy-to-use characteristics. These indel markers are expected to lay a solid foundation for further construction of inter-varietal CSSLs, with E6015-3S being the recipient parent, and for future dissection of the key genetic factors of HS tolerance in wheat. Even more, these indel markers are regarded as a useful tool for more broad applications, including genetic diversity analysis, hybrid purity testing, and MAS breeding.

## 2. Materials and Methods

### 2.1. Plant Materials

E6015-3S, a spring wheat line derived from the cross between a Chinese cultivar ‘Longmai 20’ and a Canadian cultivar Glenlea, exhibited pronounced sensitivity to heat

stress (HS) at both the seeding and adult stages [8]. ‘Bainong 207’, ‘Bainong AK58’, and ‘Liangxing 99’ are three winter wheat cultivars widely grown in northern China with strong or medium tolerance to HS [26,27]. ‘Nongda 4332’ is a *T. sphaerococcum* introgression line with semispherical grains and elevated tolerance to HS [28]. Resequencing reads of these five wheat accessions were compared with the ‘Chinese Spring’ reference genome (IWGSC RefSeq version 1.0) [29] for genomic variation calling in 2022. A panel of 16 hexaploid wheat accessions was used to amplify 1042 indel markers to test the marker’s uniqueness and polymorphism. Besides E6015-3S and ‘Chinese Spring’, the remaining 14 wheat accessions were selected based on their tolerance level in terms of HS, including a sensitive one (i.e., A8S) and 13 tolerant ones (e.g., ‘Liangxing 99’). Their phylogenetic relationships and polymorphic indel markers will provide a vital clue regarding the future construction of inter-varietal CSSLs and the identification of key genetic factors conferring HS tolerance.

## 2.2. Whole-Genome Resequencing, Sequence Alignment, and Variation Calling

The E6015-3S genome was resequenced by the BioMarker Technology Cooperation (Beijing, China) in 2019, as described in our previous study [8]. Briefly, about 10 µg of genomic DNA was used to construct a paired-end sequencing library, which was sequenced on an Illumina® HiSeq X Ten platform. The insert size was approximately 350 bp, with the read length being 150 bp. The raw reads were processed with Trimmomatic v0.36 [30], with resultant clean reads of 93.69% in the Q30 quality score and 20.14× in the genome coverage.

The whole-genome resequencing data of ‘Bainong AK58’ were collected from NCBI SRA PRJNA476679 with a sequencing depth of 8.81× [31]. For ‘Liangxing 99’ (PRJNA722149) and ‘Nongda 4332’ (PRJNA533588), paired-end sequencing was performed on the Illumina® Novaseq 6000 platform by our previous studies, with an average sequencing depth of 7.10× [32,33]. The resequencing data of ‘Bainong 207’ were collected from an unpublished project in our lab, with a sequencing depth of 6.79×. All four accessions had a read length of 150 bp and an insertion size of around 500 bp.

Genomic variation calling and quality control were performed according to procedures introduced by [34] in 2022. Briefly, raw reads were trimmed using Trimmomatic v0.36 [30] to remove poor-quality reads and adaptors. High-quality clean reads were mapped to the ‘Chinese Spring’ reference genome (RefSeq version 1.0) [29] using BWA-MEM v0.7.11 [35]. Read pairs with abnormal insert sizes (>10,000 bp) or low mapping qualities (<1) were filtered with Bamtools v2.4.168 [36]. Potential PCR duplicates were further removed using Samtools v1.3.1 [37]. Indel detection was performed by the HaplotypeCaller module of GATK v3.8.70 [38]. Indels were preliminarily filtered using the GATK VariantFiltration function with the parameters “QD < 2.0, FS > 200.0” and “ReadPosRankSum < -20.0 || DP > 30 || DP < 3”. The identified indels were further annotated with SnpEff v4.3.71 [39].

## 2.3. Genome-Wide Unique Indel Marker Development

Indels were further filtered according to E6015-3S variations in 2023, using the following parameters: E6015-3S allele as ‘1|1’ (i.e., homozygous alternative allele), mapping quality score ≥ 25, and indel size 15–75 bp. For each indel, flanking sequences of about 300 bp in length (upstream 150 bp, downstream 150 bp) were retrieved from the wheat reference genome (IWGSC RefSeq version 1.0) by using the GetSequence module in WheatOmics 1.0 (<http://wheatomics.sdau.edu.cn>, accessed on 5 December 2024) [40]. The yielded sequences were used for primer design using Primer3Plus (<https://www.primer3plus.com>, accessed on 5 December 2024) [41] with the following parameters: “product size ranges: 100–320 bp”, “primer size: min 18 bp, opt 20 bp, max 25 bp”, “Primer Tm: min 57, opt 60, max 62”, “Primer GC%: min 40%, opt 50%, max 60%”, and “Number of return: 5 primer

pairs". Obtained primer pairs were further checked for genome-wide uniqueness with the "Specificity Check" function in the PrimerServer module from WheatOmics 1.0 [40] using the following parameters: "product size: 100–500 bp" and "checking database: 'Chinese Spring' IWGSC RefSeq version 1.0". Only primer pairs with one single amplicon in the wheat genome were kept and used for further analysis (Figure 1).

#### Step 1: whole-genome resequencing, sequence alignment, and variation calling

Material, heat stress sensitive wheat line E6015-3S; insert size, ~ 350 bp; sequencing platform, Illumina® HiSeq X Ten; read length, 150 bp; > Q30 percentage, 93.69%; genome coverage, ~ 20x; reference genome, IWGSC RefSeq version 1.0; read alignment, BWA-MEM; indel detection, GATK HaplotypeCaller; indel filtration, GATK VariantFiltration.

#### Step 2: Indel selection and primer design

Indel selection, 15–75 bp in length, E6015-3S's genotype 1|1, mapping quality score  $\geq 25$ ; flanking sequences ~ 150 bp upstream and downstream the InDel, obtained by the GetSequence module in WheatOmics 1.0; primer design in Primer3Plus, with 150–250 bp in amplicon size, 18–25 bp in primer length, 57–62 °C in melting temperature, 40–60% in GC content, 5 primer pairs to return.

#### Step 3: genome-wide uniqueness check

Uniqueness check by the PrimerServer module in WheatOmics 1.0, with product size 100–500 bp, checking database IWGSC RefSeq version 1.0; for those ideal primer pairs with a single hit, check uniqueness against databases including 'Kenong 9204', 'Bainong AK58', 'Zang 1817', Fielder and 12 hexaploid wheat accessions from the 10+ Wheat Genome; PCR detection, reaction volume 15  $\mu$ L, annealing temperature 60 °C, 35 cycles, separation on 1.0% agarose gel or 8.0% polyacrylamide gel.

**Figure 1.** Schematic representation of the actions taken to develop genome-wide unique indel markers in wheat.

#### 2.4. Uniqueness Check of Indel Markers Among Sequenced Wheat Genomes

To check their uniqueness in the wheat genome, primer sequences were blasted against another 17 versions of sequenced wheat genomes in 2024. These include an improved genome version of 'Chinese Spring' (IWGSC RefSeq version 2.1) [42], two Chinese winter wheat cultivars 'Bainong AK58' and 'Kenong 9204' [43,44], one Tibetan semi-wild wheat accession 'Zang 1817' [45], one transformation-amenable common wheat cultivar Fielder [46], and another 12 sequenced genomes from the 10+ Wheat Genomes [47]. A genome uniqueness check of primer pairs was performed with the "Specificity Check" function in the PrimerServer module from WheatOmics 1.0 [40] using "product size: 100–500 bp" as the filtering parameter.

#### 2.5. DNA Extraction and PCR Amplification

Genomic DNA was extracted from young leaves in 2023 using standard CTAB methods [48]. The extracted DNA concentration was measured using a microvolume spectrophotometer and adjusted to a final concentration of 30 ng/ $\mu$ L. PCR was performed in a reaction volume of 15  $\mu$ L, which included approximately 60 ng of template DNA, 7.5  $\mu$ L of 2 $\times$  Taq Plus Master Mix (Novoprotein, Suzhou, Jiangsu, China), 1.5  $\mu$ L of 10 nM primer, and ddH<sub>2</sub>O. Thermocycling was started at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 10 min. The PCR products were separated by 1.0% agarose gel or 8.0% polyacrylamide gel depending on different indel sizes and polymorphism ratios. E6015-3S and 'Chinese Spring' were used as controls during the PCR amplification and the following allele deciphering.

## 2.6. Phylogenetic Analysis

A phylogenetic analysis was performed in 2024 according to procedures introduced by [49]. Briefly, bands on polyacrylamide gels visualized by silver staining were scored as present (1) or absent (0). These data were collected and analyzed with the numerical taxonomy multivariate analysis system software package (NTSYS pc 2.10) [50], a classical and popular software for analyzing genetic diversity. A similar matrix was constructed using indel data based on Dice's coefficient, which considers only one-to-one matches between two taxa for similarity [51]. Based on the obtained similarity matrix, a dendrogram was constructed by the Unweighted Pair-Group Method using arithmetic Averages (UPGMA) to determine the genetic relationships among studied wheat accessions.

## 3. Results

### 3.1. Primer Design and Uniqueness Check

To develop a set of practical markers for the heat-sensitive wheat genotype E6015-3S, its resequencing data were blasted against the 'Chinese Spring' reference genome (IWGSC RefSeq version 1.0). A total of 19,970 indels meeting the following criteria were selected: (i) indel size 15–75 bp; (ii) mapping quality score  $\geq 25$ ; (iii) E6015-3S allele as '1|1' (i.e., homozygous alternative allele). Targeting 18,447 indels, with a ratio of 92.37% of selected indels, we designed 89,542 pairs of primers using the 'Chinese Spring' reference genome as a template. Each indel has an average of 4.85 pairs of primer. The average indel density is 1.31 indels per Mbp of DNA (Table 1). The highest occurs on chromosomes 2B, 3B, 5B, and 6B (1.78–2.24 indels/Mbp), and the lowest occurs on chromosomes 4D, 5D, 6D, and 7D (0.53–0.77 indels/Mbp). This implies that the density of targeted indels varies among different subgenomes in descending order, i.e., from B down to A and D.

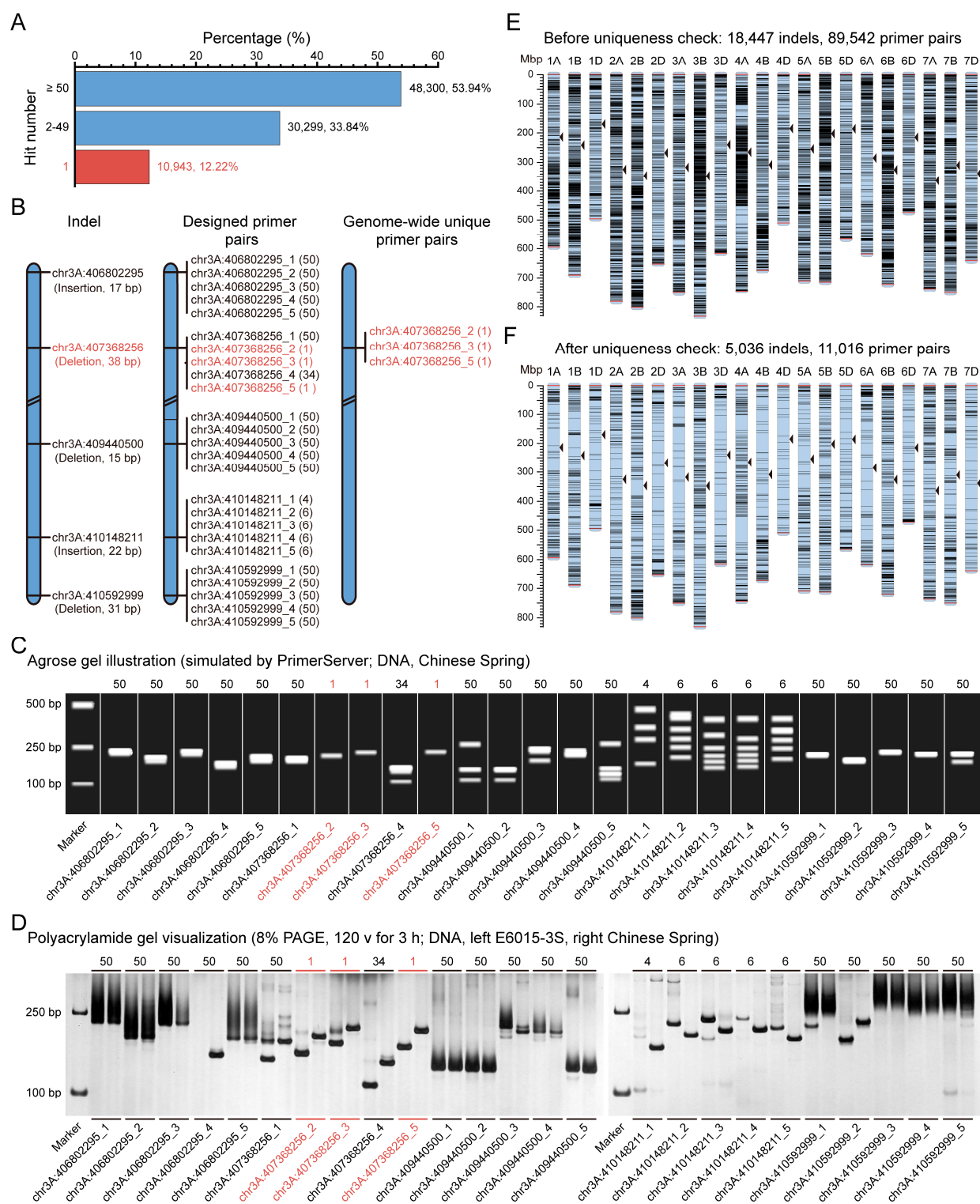
To enhance their practicality, primer sequences were subsequently mapped back to the 'Chinese Spring' reference genome, and more than half (53.94%, 48,300) of primer pairs exhibited 50 or more hits in the wheat genome (Figure 2A). This result indicates that a considerable proportion of indels locate within repetitive genomic regions, and primer pairs targeting these indels most likely amplify overlapped bands, hence being useless in genetic studies of wheat. To test and prove this speculation, we randomly synthesized and amplified 25 primer pairs from five neighboring indels on chromosome 3A (Figure 2B,C). PCR products were separated by 8.0% polyacrylamide gel electrophoresis. Nearly all primer pairs with 50 hits in the wheat genome yielded overlapped bands or blurry 'shadow bands', whereas primer pairs with a unique hit yielded a single and polymorphic band (Figure 2D).

A total of 11,016 specific primer pairs were identified on 21 chromosomes, with a rate of 12.30% of the designed 89,542 primer pairs (Figure 2E,F). Besides the 10,943 (12.22%) genome-wide unique primer pairs (Figure 2A), another 73 (0.08%) primer pairs were also included, which amplify dispersed bands with relatively big gaps (Table S1). These 11,016 primer pairs targeted 5036 indels in total, with an average of 2.19 primer pairs per indel. Indel density along the whole genome was about 0.36 indels per Mbp (Table 1). The highest occurred on chromosomes 2B, 5B, and 6B (0.48–0.53 indels/Mbp), whereas the lowest occurred on chromosomes 4D, 5D, and 7D (0.12–0.24 indels/Mbp). The sequences of these primer pairs, as well as the positions and amplicon lengths, are presented in Table S2.

**Table 1.** Designed indel markers before and after the uniqueness check.

| Chr. | Before Uniqueness Check |                 |                   |                |                     | After Uniqueness Check |                    |                   |                       |                     |
|------|-------------------------|-----------------|-------------------|----------------|---------------------|------------------------|--------------------|-------------------|-----------------------|---------------------|
|      | Indel No.               | Primer Pair No. | Primer Pair/Indel | Coverage (Mbp) | Density (Indel/Mbp) | Indel No.              | Primer Pair No.    | Primer Pair/Indel | Coverage (Mbp)        | Density (Indel/Mbp) |
| 1A   | 755                     | 3724            | 4.93              | 590.52         | 1.28                | 230<br>(30.46%) *      | 549<br>(14.74%)    | 2.39<br>(48.45%)  | 578.43<br>(97.95%)    | 0.40<br>(31.10%)    |
| 2A   | 914                     | 4379            | 4.79              | 780.45         | 1.17                | 221<br>(24.18%)        | 513<br>(11.72%)    | 2.32<br>(48.42%)  | 779.02<br>(99.82%)    | 0.28<br>(24.22%)    |
| 3A   | 782                     | 3883            | 4.97              | 748.70         | 1.04                | 211<br>(26.98%)        | 509<br>(13.11%)    | 2.41<br>(48.54%)  | 743.54<br>(99.31%)    | 0.28<br>(27.17%)    |
| 4A   | 1252                    | 5854            | 4.68              | 741.01         | 1.69                | 238<br>(19.01%)        | 480<br>(8.20%)     | 2.02<br>(43.20%)  | 739.87<br>(99.85%)    | 0.32<br>(19.04%)    |
| 5A   | 862                     | 4115            | 4.77              | 706.56         | 1.22                | 247<br>(28.65%)        | 519<br>(12.61%)    | 2.10<br>(43.99%)  | 703.87<br>(99.62%)    | 0.35<br>(28.76%)    |
| 6A   | 589                     | 3562            | 6.05              | 617.88         | 0.95                | 242<br>(41.09%)        | 559<br>(15.69%)    | 2.31<br>(38.20%)  | 617.52<br>(99.94%)    | 0.39<br>(41.11%)    |
| 7A   | 1092                    | 5141            | 4.71              | 736.50         | 1.48                | 338<br>(30.95%)        | 709<br>(13.79%)    | 2.10<br>(44.61%)  | 734.81<br>(99.77%)    | 0.46<br>(31.02%)    |
| 1B   | 979                     | 4727            | 4.83              | 688.34         | 1.42                | 264<br>(26.97%)        | 577<br>(12.21%)    | 2.19<br>(45.36%)  | 687.09<br>(99.82%)    | 0.38<br>(27.02%)    |
| 2B   | 1536                    | 7178            | 4.67              | 799.22         | 1.92                | 421<br>(27.41%)        | 990<br>(13.79%)    | 2.35<br>(50.29%)  | 798.49<br>(99.91%)    | 0.53<br>(27.43%)    |
| 3B   | 1566                    | 7486            | 4.78              | 827.34         | 1.89                | 341<br>(21.78%)        | 699<br>(9.34%)     | 2.05<br>(42.88%)  | 821.63<br>(99.31%)    | 0.42<br>(21.93%)    |
| 4B   | 809                     | 4201            | 5.19              | 672.95         | 1.20                | 191<br>(23.61%)        | 402<br>(9.57%)     | 2.10<br>(40.44%)  | 672.65<br>(99.96%)    | 0.28<br>(23.62%)    |
| 5B   | 1271                    | 6123            | 4.82              | 712.56         | 1.78                | 339<br>(26.67%)        | 765<br>(12.49%)    | 2.26<br>(46.91%)  | 712.32<br>(99.97%)    | 0.48<br>(26.68%)    |
| 6B   | 1612                    | 7657            | 4.75              | 720.44         | 2.24                | 379<br>(23.51%)        | 754<br>(9.85%)     | 1.99<br>(41.89%)  | 720.44<br>(100.00%)   | 0.53<br>(23.51%)    |
| 7B   | 1245                    | 6010            | 4.83              | 749.58         | 1.66                | 323<br>(25.94%)        | 666<br>(11.08%)    | 2.06<br>(42.67%)  | 749.57<br>(100.00%)   | 0.43<br>(25.94%)    |
| 1D   | 501                     | 2432            | 4.85              | 493.78         | 1.01                | 175<br>(34.93%)        | 423<br>(17.39%)    | 2.42<br>(49.85%)  | 492.41<br>(99.72%)    | 0.36<br>(35.03%)    |
| 2D   | 678                     | 3180            | 4.69              | 649.86         | 1.04                | 214<br>(31.56%)        | 497<br>(15.63%)    | 2.32<br>(49.46%)  | 644.97<br>(99.25%)    | 0.33<br>(31.80%)    |
| 3D   | 600                     | 2839            | 4.73              | 613.45         | 0.98                | 216<br>(36.00%)        | 495<br>(17.44%)    | 2.29<br>(48.40%)  | 611.24<br>(99.64%)    | 0.35<br>(36.13%)    |
| 4D   | 269                     | 1403            | 5.22              | 507.27         | 0.53                | 60<br>(22.30%)         | 121<br>(8.62%)     | 2.02<br>(38.73%)  | 505.26<br>(99.60%)    | 0.12<br>(22.39%)    |
| 5D   | 365                     | 1864            | 5.11              | 564.74         | 0.65                | 133<br>(36.44%)        | 282<br>(15.13%)    | 2.12<br>(41.51%)  | 562.98<br>(99.69%)    | 0.24<br>(36.55%)    |
| 6D   | 366                     | 1773            | 4.84              | 472.67         | 0.77                | 158<br>(43.17%)        | 315<br>(17.77%)    | 1.99<br>(41.08%)  | 472.50<br>(99.97%)    | 0.33<br>(43.18%)    |
| 7D   | 404                     | 2011            | 4.98              | 635.22         | 0.64                | 95<br>(23.51%)         | 192<br>(9.55%)     | 2.02<br>(40.58%)  | 633.93<br>(99.80%)    | 0.15<br>(23.56%)    |
| All  | 18,447                  | 89,542          | 4.85              | 14,028.99      | 1.31                | 5036<br>(27.30%)       | 11,016<br>(12.30%) | 2.19<br>(45.12%)  | 13,982.54<br>(99.67%) | 0.36<br>(27.39%)    |

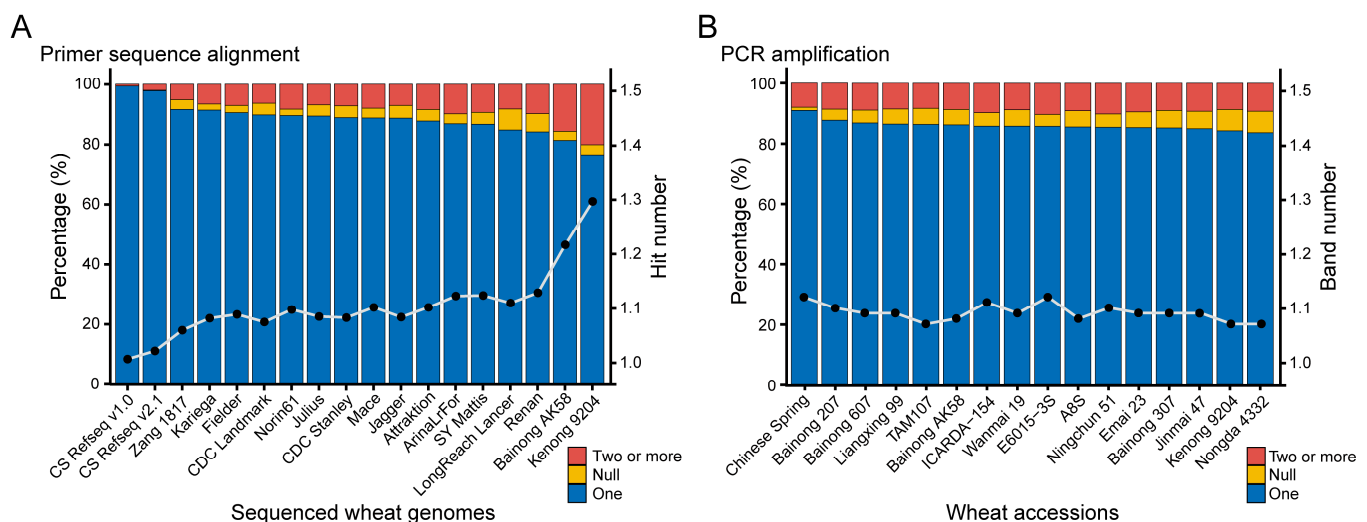
\* The percentages in parenthesis indicate the remaining proportions after the uniqueness check.



**Figure 2.** Development of 11,016 genome-wide unique primer pairs targeting 5036 indel sites. (A) Sequence alignment of 89,542 primer pairs against the ‘Chinese Spring’ reference genome (IWGSC RefSeq version 1.0), which was performed with the PrimerServer module in WheatOmics 1.0 [40]. (B–D) The agarose gel illustration (simulated by the PrimerServer module in WheatOmics 1.0) and polyacrylamide gel visualization (8% PAGE, 120 v for 3 h) for a few primer pairs. Primer pairs with multiple hits (in black) tended to yield overlapped or blurry shadow bands, whereas the ones with a unique hit (in red) tended to produce a single and polymorphic band. (E,F) The indel sites targeted by primer pairs along each chromosome before (E) and after (F) the uniqueness check. Detailed information is presented in Table S2.

### 3.2. Indel Marker's Uniqueness in the Wheat Genome

To check the specificity of the indel markers in the wheat genome, primer sequences were blasted against 18 versions of sequenced wheat genomes, including the two versions of 'Chinese Spring' (IWGSC RefSeq versions 1.0 and 2.1), 'Kenong 9204', 'Bainong AK58', 'Zang 1817', Fielder and 12 versions from the 10+ Wheat Genomes (Table S3). In the IWGSC RefSeq version 2.1 of 'Chinese Spring', 10,775 primer pairs produced a single hit, a ratio of 97.81% of the blasted 11,016 primer pairs, which is almost the same as that (99.34%) in IWGSC RefSeq version 1.0 (Figure 3A). This ratio among the rest genomes is slightly lower, from 76.18% (8392) in 'Kenong 9204' to 91.58% (10,089) in 'Zang 1817' (with an average of 87.25% (9611.50)), because of an elevated proportion of the 'two and more' alignment (Table S4). As for the null alignment, its ratio is quite similar among tested genomes, from 2.02% (223) in Kariaga to 4.17% (460) in Jagger, but with exceptions of the two versions of 'Chinese Spring' (version 1.0, 0.00%, 0; version 2.1, 0.17%, 19), 'LangReach Lancer' (7.01%, 772) and Renan (6.10%, 672). Accordingly, the average hit number of the 11,016 primer pairs among tested genomes varied from 1.01 to 1.30 (Table S3; Figure 3A).

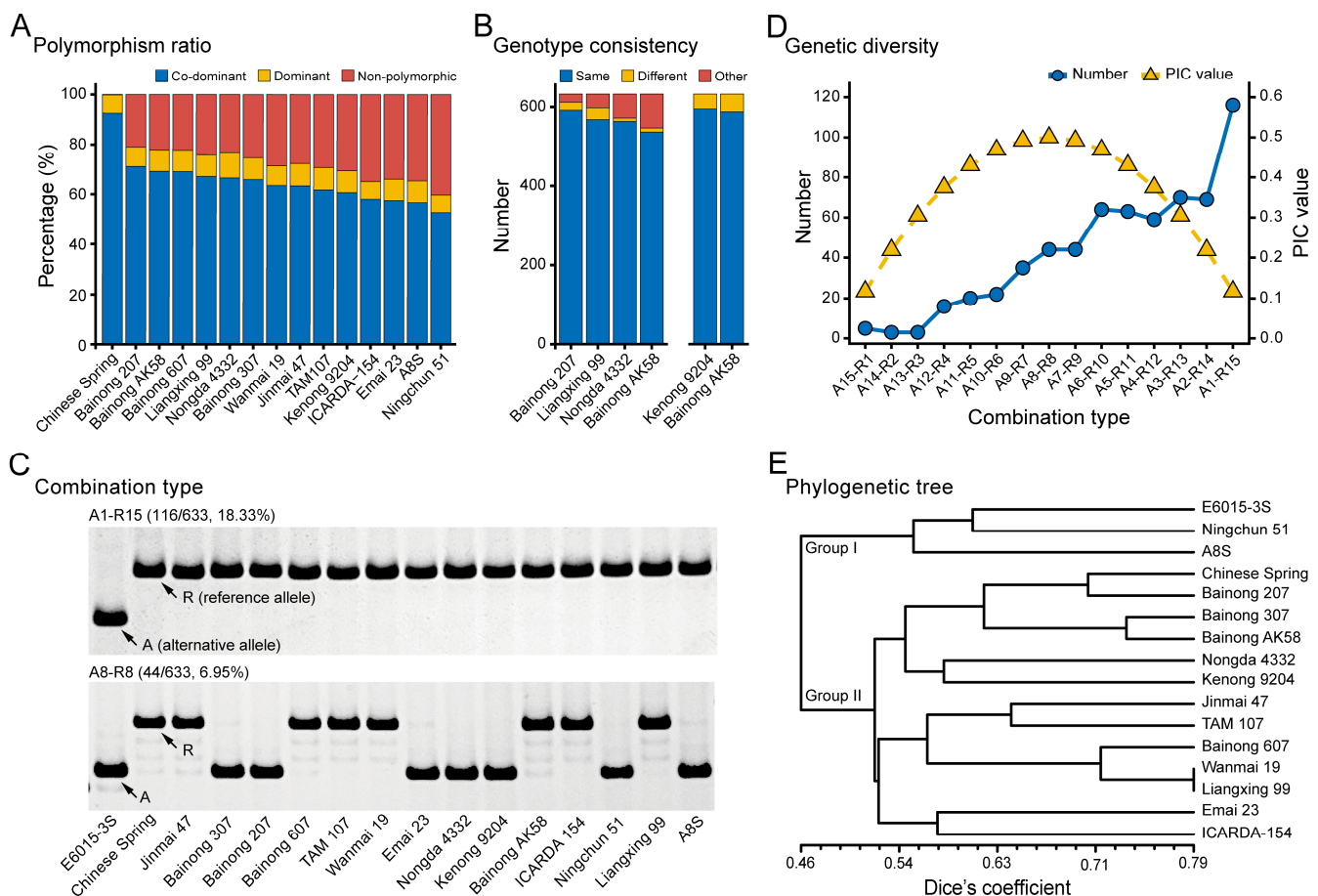


**Figure 3.** Uniqueness of indel markers among 18 versions of sequenced wheat genomes and 16 wheat accessions. **(A)**, The specificity check of the 11,016 primer pairs among 18 versions of sequenced wheat genomes was performed with the PrimerServer module in WheatOmics 1.0 [40]. **(B)**, PCR products were separated on 8% polyacrylamide gels. A total of 1042 (97.75%) pairs amplified clear bands, and their band numbers among tested wheat accessions were counted. Detailed information is presented in Tables S3–S6.

To further verify the specificity of indel markers in the wheat genome, we synthesized 1066 primer pairs and amplified them using 16 hexaploid wheat accessions. PCR products were separated on 8% native PAGE gels. A total of 1042 (97.75%) primer pairs amplified clear bands, and their band numbers among tested wheat accessions were recorded (Table S5). In Chinese Spring, 948 primer pairs amplified a single band, a ratio of 90.98% of the analyzed 1042 primer pairs (Figure 3B). This ratio in other wheat accessions was slightly lower, ranging from 83.59% (871) in 'Nongda 4332' to 87.72% (914) in 'Bainong 207' (with an average of 85.67% (892.73)), due largely to an elevated number of null amplicon (Table S6). The ratio of 'two or more' bands is comparable among tested accessions, from 7.97% (83) in Chinese Spring to 10.36% (108) in E6015-3S. Consequently, the average band number among tested accessions is quite similar, varying from 1.07 to 1.12 (Table S5; Figure 3B).

### 3.3. Indel Marker's Polymorphism Characteristic

To test the cross-cultivar polymorphism, the allele information of the 1042 indel markers among 16 wheat accessions was collected and analyzed. Between ‘Chinese Spring’ and E6015-3S, 964 co-dominant (CD) and 76 dominant (D) markers were observed, representing a polymorphism ratio as high as 99.81% (1040) (Figure 4A). The polymorphism ratios between the other individual wheat accessions and E6015-3S were also calculated, and they varied from 59.69% (622, with 549 CD and 73 D) in ‘Ningchun 51’ to 78.98% (823, with 740 CD and 83 D) in ‘Bainong 207’ (Table S7). Fifty-four Xbarc SSR markers (from *Xbarc1025* to *Xbarc1119*) developed by Song et al. [52] were randomly selected to amplify the 16 wheat accessions with an aim to compare the newly developed indel markers with an existing marker system. Results showed that nine markers yielded blurry shadow bands, while the remaining 45 markers produced clear bands and were used to calculate polymorphism ratios. Results showed that 13.33% to 28.89% of Xbarc SSR markers were polymorphic between 15 individual wheat accessions and E6015-3S (Table S8), which were much lower compared to that in indel markers.



**Figure 4.** Polymorphism of indel markers among 16 wheat accessions. (A), Polymorphism ratio between 15 individual wheat accessions and E6015-3S. (B), Genotype consistency in the allele information collected from different ways: left, between PCR amplification and whole-genome resequencing; right, between PCR amplification and reference genome sequence. (C,D), The 15 combination types formed within the 633 indel markers, which yielded only two separate alleles, i.e., the reference (R) allele from ‘Chinese Spring’ and the alternative (A) allele from E6015-3S. (E), Phylogenetic tree showing the genetic relations among the 16 wheat accessions, which was constructed using the mentioned 633 indel markers. Detailed information is presented in Tables S7 and S9–S12.

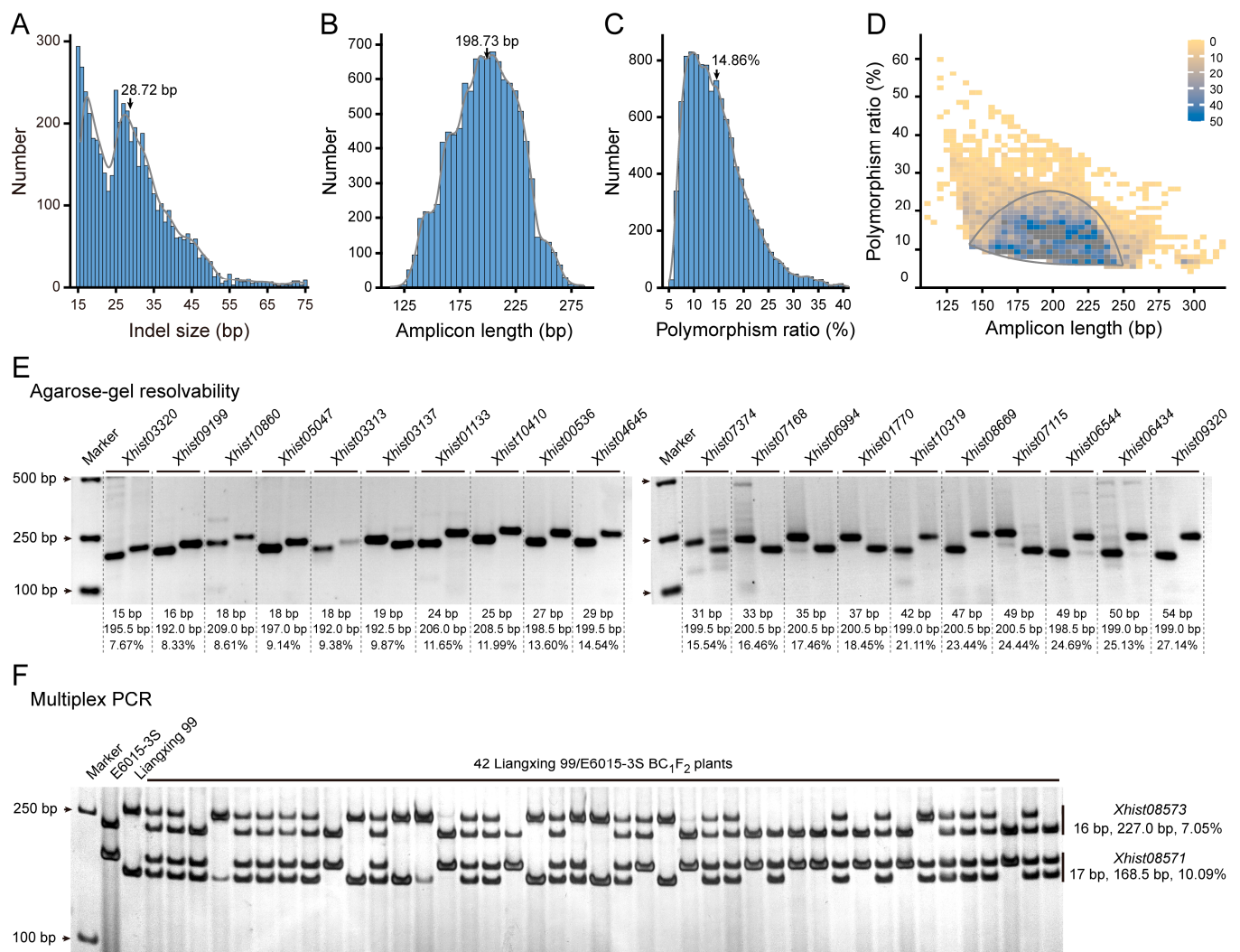
Those 633 indel markers producing only two separate alleles, i.e., the reference (R) allele as ‘Chinese Spring’ and the alternative (A) allele as E6015-3S, were used to perform the following analyses. Regarding the genotype consistency between PCR amplification and genome resequencing, 537 (84.83%) to 592 (93.52%) of the tested indel markers exhibited accordant genotypes in ‘Bainong 207’, ‘Liangxing 99’, ‘Nongda 4332’, and ‘Bainong AK58’, while 21 (3.32%) to 86 (13.59%) failed in comparison due to a lack of resequencing data (Table S9; Figure 4B). As for the genotype consistency between PCR amplification and reference genome sequences, 595 (94.00%) and 588 (92.89%) of tested indel markers yielded agreeing genotypes in ‘Kenong 9204’ and ‘Bainong AK58’, respectively (Table S10; Figure 4B).

Based on the number of A and R alleles, these 633 indel markers were further classified into 15 combination types. Nine types (from A12-R4 to A4-R12) had polymorphism information content (PIC) values that were over 0.35 and covered 367 indel markers in total (Table S11; Figure 4C,D), and these were used for further phylogenetic analysis. The two heat-sensitive wheat lines (i.e., E6015-3S and A8S) were clustered together in Group I, whereas several heat-tolerant wheat lines gathered in Group II, such as ‘Liangxing 99’, ‘Bainong 207’, ‘Bainong AK58’, ‘Nongda 4332’, and ‘TAM 107’ (Table S12; Figure 4E). Our results indicated that these indel markers possessed good polymorphism among various wheat accessions, hence representing a reliable and accurate genotyping platform for wheat genetics studies.

### 3.4. Indel Marker’s Easy-to-Use Characteristic

A thorough investigation of the 11,016 primer pairs was performed, examining the indel sizes, amplicon sizes, and polymorphism ratios (Table S2). Among their targeted 5036 indels, the ones (4709, 93.51%) ranging from 15 to 46 bp in size were predominant (Figure 5A). The amplicon sizes of primer pairs, i.e., the mean length of amplified fragments from ‘Chinese Spring’ and E6015-3S, were highly enriched (10,226, 92.83%) in ranges from 141 to 250 bp (Figure 5B). Polymorphism ratios of primer pairs, which were calculated through dividing the amplicon size by the indel size, clustered in abundance (10,346, 93.92%) within the category from 6.0 to 25.0% (Figure 5C). Accordingly, a clear tendency was observed that most primer pairs have amplicon lengths from 141 to 250 bp and polymorphism ratios from 6.0 to 25.0% (Figure 5D).

To highlight their utilization, representative primer pairs were examined for resolvability on agarose gels. The 20 primer pairs used for this analysis had amplicon sizes ranging from 192.0 to 209.0 bp, indel sizes ranging from 15 to 54 bp, and polymorphism ratios ranging from 7.67 to 27.14% (Figure 5E). After electrophoresis for 2.5 h at 160 v in 1.0% agarose gel, all 20 primer pairs yielded unambiguous polymorphic bands in E6015-3S and ‘Chinese Spring’. Furthermore, two primer pairs with varying amplicon sizes and polymorphism ratios were randomly selected for multiplex PCR. A mixture of a DNA template, two primer pairs, and 2× Taq Plus Master Mix (Novoprotein, Suzhou, Jiangsu, China) was amplified by 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C. After polyacrylamide gel electrophoresis, both of the two markers yielded clear polymorphic bands among E6015-3S, ‘Liangxing 99’, as well as their 42 BC<sub>1</sub>F<sub>2</sub> descendants (Figure 5F).



**Figure 5.** Easy-to-use characteristic of developed indel markers. (A–D), Distributions of indel size, amplicon size, and polymorphism ratio of the 11,016 primer pairs, which target 5036 indels in the wheat genome. The numbers close to the arrows indicate the mean values. (E), Experimental validation of marker polymorphism in 1% agarose gel (160 v, 2.5 h). DNA samples are E6015-3S (left) and ‘Chinese Spring’ (right). The numbers indicate indel size (top), amplicon size (middle), and polymorphism ratio (bottom), respectively. (F), Multiplex PCR of two randomly selected primer pairs. PCR products were separated on 8% polyacrylamide gel (150 v, 2 h). The numbers indicate indel size (left), amplicon size (middle), and polymorphism ratio (right), respectively. Marker, DL2000.

#### 4. Discussion

Chromosome segment substitution lines (CSSLs) have long been important tools in plant genome research, especially in the detection of QTLs and causal genes for crop improvement [15]. Recently, SNP markers have been made available on high-throughput solid- and liquid-phase genotyping arrays [53,54]. However, these arrays are inflexible in their design and time-consuming in regard to generating an informative result, making them unsuitable for long-term MAS during CSSL development [55]. SSRs were the most preferred for tracking substituted segments in diploid genomes like rice because of their ease of genotyping in large populations [11]. However, in hexaploid bread wheat, SSRs proved less suitable for tackling this task because of their multi-locus and redundancy characteristics [25]. Here, we report the development of 11,016 genome-wide unique primer pairs targeting 5036 indel sites (Tables 1 and S2; Figure 2F), which represents an ideal genotyping platform for the inter-varietal CSSL construction in hexaploid wheat.

Inter-varietal CSSL construction in wheat has lagged behind other species, partially owing to the challenges of a huge, hexaploid, and complex genome that contains more than 85% repetitive DNA [29]. In the resequencing era, indel variations can be obtained easily by mapping the resequencing reads against a reference genome [34], but wheat indels recognized this way have a great possibility of being located within repetitive genomic regions. In our study, 87.78% (78,599) of the designed 89,542 primer pairs were aligned to multiple loci, and more than half (53.94%, 48,300) of them exhibited 50 or even more hits in the wheat genome (Figure 2A,B). Nearly all synthesized primer pairs with 50 or more hits amplified overlapped bands or blurry ‘shadow bands’ (Figure 2D), hence being useless in genotyping analyses. To overcome the problem of multi-locus, special emphasis was placed on exploring genome-wide unique indel markers in this study, which proved to be a highly effective methodology. We blasted the sequences of 11,016 primer pairs against 18 sequenced wheat genomes and found that 76.18 to 99.34% of them yielded a single hit in an individual genome (Table S3; Figure 3A). Furthermore, we amplified 1042 primer pairs among 16 wheat accessions and found that 83.59 to 90.98% of them amplified a single band in an individual accession (Table S5; Figure 3B). Consequently, 59.69 to 99.81% of the tested 1042 primer pairs were polymorphic between E6015-3S and the other 15 individual accessions (Table S7; Figure 4A), a ratio much higher than reported DNA markers (Table S8) [52,56].

Short indel polymorphisms of less than 15 bp require electrophoresis in polyacrylamide gels, which is labor-intensive and time-consuming [57]. In comparison, long indel polymorphisms of over 15 bp in size are resolvable through agarose gel electrophoresis, which needs simple requirements and greatly enhances detection efficiency at a moderate cost and on a large scale [58,59]. In this study, most of the designed 11,016 primer pairs have indel sizes ranging from 15 to 46 bp, amplicon sizes ranging from 141 to 250 bp, and polymorphism ratios ranging from 6.0 to 25.0% (Table S2; Figure 5B,D). Furthermore, a shift from uniplex to multiplex PCR will allow for the simultaneous amplification of multiple markers [60,61] and is a second way to accelerate marker-assisted selection during wheat CSSL development. We randomly selected two indel markers for multiplex PCR and found that both of them yielded clear polymorphic bands among 42 BC<sub>1</sub>F<sub>2</sub> plants as well as their parental lines (Figure 5F). Empirically, about 15 to 25 DNA markers that are evenly distributed along a chromosome are needed during inter-varietal CSSL development [62,63]. Since the number of designed primer pairs on each wheat chromosome varied from 121 to 990, with an average of 524.57 (Tables 1 and S2; Figure 2F), appropriate markers with large indel sizes, high polymorphism ratios, and suitable amplicon lengths could be selected to accommodate agarose gel electrophoresis and multiplex PCR, as illustrated in Figure 5E,F.

Our ongoing research is trying to construct a few series of inter-varietal CSSLs, using E6015-3S (HS sensitive) [8] as the recipient parent and several HS-tolerant varieties as donor parents, which were pre-analyzed for polymorphism and genetic similarity in this study. According to the obtained results, 59.59 to 78.98% of tested 1042 indel markers were polymorphic between an individual wheat accession and E6015-3S (Table S7; Figure 4A). The phylogenetic dendrogram categorized the accessions into two groups with 50% similarity (Table S12; Figure 4E), with E6015-3S and A8S (another HS sensitive genotype) [10] being clustered in Group I and several HS-tolerant accessions, such as ‘Liangxing 99’ [26,27] ‘Bainong AK58’ [26], ‘Bainong 207’ [27], ‘Nongda 4332’ [28], and ‘TAM 107’ [64] being gathered in Group II. Collectively, the newly developed genome-wide unique indel markers have laid a solid foundation for the development of inter-varietal CSSLs, which are key materials for studying the quantitative inheritance of HS tolerance in wheat.

## 5. Conclusions

In this study, we developed 11,016 primer pairs targeting 5036 indel sites for a HS sensitive genotype, with an average density of 0.36 indels per Mbp along the whole genome. They were genome-wide unique, highly polymorphic, and easy to use, representing an ideal PCR-based marker system for a wide range of applications, including, but certainly not limited to inter-varietal CSSL development, gene mapping and marker-assisted breeding in wheat. Phylogenetic relationships and polymorphic indel markers would pave a way to the future development of HS-tolerant CSSLs in wheat, based on which key genes controlling HS tolerance in wheat could be cloned and utilized to ensure global food safety.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy15010169/s1>, Table S1: The 73 primer pairs that amplify dispersed bands with relatively big gaps; Table S2: The positions, primer sequences, indel sizes, amplicon sizes, and polymorphism ratios of the 11,016 genome-wide unique primer pairs; Table S3: Detailed information about primer sequence alignment of the 11,016 indel markers among 18 sequenced wheat genomes; Table S4: Statistics of hit number of the 11,016 indel markers among 18 sequenced wheat genomes; Table S5: Detailed information about PCR amplification of the 4012 indel markers among 16 wheat accessions; Table S6: Statistics of PCR band number of the 1042 indel markers among 16 wheat accessions; Table S7: Statistics of polymorphism ratios of the 1042 indel markers between 15 individual wheat accessions and E6015-3S; Table S8: Statistics of polymorphism ratios of the 45 SSR markers between 15 individual wheat accessions and E6015-3S; Table S9: Detailed information and statistics of genotype consistency between PCR amplification and genome resequencing; Table S10: Detailed information and statistics of genotype consistency between PCR amplification and reference genome sequence; Table S11: Statistics of polymorphism information content (PIC) values, marker numbers, and percentages of the 15 combination types; Table S12: Genetic similarity coefficients among the 16 wheat accessions.

**Author Contributions:** H.Z. and Z.N. perceived the project. H.Z., K.X., M.W., Z.W. and Z.C. took part in primer design, uniqueness check, and genotype detection analysis. H.Z., A.L., A.H., X.X. and L.C. conducted bioinformatic and phylogenetic analyses. H.Z. conducted data analysis, validation, and visualization. M.L. and X.O. assisted in reviewing the manuscript. H.Z. and Z.N. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflicts of interest.

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