Detection of QTLs for Outcrossing-Related Traits in CSSL Population Derived from Primitive Japonica Accession Ludao in the Genetic Background of *O. sativa* spp. Japonica Restorer C-bao using RSTEP-LRT Method

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**Abstract:** The outcrossing traits in rice (*Oryza sativa* L.) affect the yield of hybrid seed production. Using a cytoplasmic male sterile (CMS) line with good outcrossing traits, such as short flag leaf length (FLL), narrow flag leaf width (FLW), wide flag leaf angle (FLA), and elongated panicle neck length (PNL), for hybrid rice seed production, it is possible to avoid the procedure of cutting flag leaves and make the supplementary pollination feasible by machine. In this study, a *japonica* restorer C-bao as the receptor parent and a primitive *japonica* accession Ludao as the donor parent were used to construct a chromosome segment substitution line (CSSL) population. The CSSL population was used to detect quantitative trait loci (QTLs) for the four outcrossing traits using a likelihood ratio test based on the stepwise regression (RSTEP-LRT) method. The CSSL population constructed consisted of 163 lines covering 90.7% of the donor genome. Among the seven QTLs detected in the CSSL population, four QTLs were detected in both years. *qFLL*-4 explained 6.70% of the two-year-averaged phenotypic variance, and the alleles from Ludao decreased FLL 5.1 cm. *qFLL*-1.1 and *qFLL*-1.2 explained 7.85% and 21.29% of the 2-year-averaged phenotypic variance respectively, and the alleles from Ludao increased FLL 17.38° and 31.50°. *qPNL*-8 explained 8.87% of the 2-year-averaged phenotypic variance, and the alleles from Ludao increased PNL 4.44 cm. These favorable alleles identified could be used to improve the outcrossing traits of parents for hybrid rice seed production in rice.

**Keywords:** *Oryza sativa*; flag leaf length; flag leaf width; flag leaf angle; panicle neck length; QTLs

1. Introduction

Rice (*Oryza sativa* L. and *Oryza glaberrima* Steud.) is the leading food crop for over 3 billion people. It is grown on 160 million hectares per year in 117 countries with an average production of...
approximately 4.4 tons per hectare [1]. The constant growth in the human population and a decline in cultivation land has left no choice but to increase the rice crop production per unit area and ensure food security for billions of people around the world [2,3]. Among various strategies to increase rice yield, utilization of heterosis is an effective tool for increasing yield production [4,5]. However, every year, F1 hybrid seed is required for the utilization of heterosis. In the hybrid rice seed production process, farmers need to manually cut one-third to one-half of the flag leaf in the female parent plant at the early heading stage to facilitate cross-pollination. It is not only a labor-intensive procedure, but professional skills are also needed to avoid any damage to young panicles. Using a cytoplasmic male sterile (CMS) lines with good outcrossing traits, such as a short flag leaf length, wide angle, and elongated panicle neck length, can not only avoid the procedure of cutting flag leaves but also make the hybrid rice seed production by machine feasible.

The flag leaf trait is a major determinant of plant architecture with complex inheritance. To date, 65 QTLs have been noticed for FLL, including 15, 4, 10, 5, 3, 8, 4, 2, 3, 5, 2, and 4 on chromosome 1 to 12, respectively [6,7] (http://www.gramene.org). Yan et al. [8] detected QTLs for the flag leaf length trait on chromosome 4 in a doubled haploid population derived from IR64/Aluzena cross and showed that these regions may have genetic factors controlling flag leaf development.

At least 35 QTLs controlling flag leaf width have been identified, with 5, 4, 4, 3, 4, 1, 5, 2, 1, 1, 2, and 3 on chromosome 1 to 12, respectively [6,7]. A mutant gene—narrow-leaf 7 (nal7)—has been isolated by the map-based cloning method [9–12] (http://www.gramene.org). Nal7, located on chromosome 3, encodes a flavin containing mono-oxygenase, and a spontaneous mutation in this gene significantly decreased leaf width and altered indole-3-acetic acid content in leaf [10]. A recent study showed that nal1-2 and nal1-3 at NAL1 locus reduced 50% of leaf width and it was caused by affecting cell division at the initial stage of leaf growth [9].

In rice, the angle between the main stem and the flag leaf is one of the rice plant type indicators. The flag leaf angle (FLA) in rice is controlled by multiple genes having some major and minor effects on the trait. Seventy-nine QTLs for FLA have been detected and distributed on all 12 rice chromosomes in previous reports [13–19]. Among these detected QTLs, 10, 7, 9, 4, 6, 7, 5, 9, 4, 5, 7, and 6 were noticed on chromosome 1 to 12, respectively, with the percentage of phenotypic variance explained ranging from 4.2% to 48.29% [7,19]. At present, the biological mechanism of the rice leaf angle has made great progress. Brassinosteroid (BR) signal transduction through the expression of key genes causes leaf angle inclination. The overexpression of DLT (dwarf and low-tilling) and GSK2 (glycogen synthetase kinase 2), the key genes of the BR signaling pathway, increased the leaf angle [20–22]. The gene LPA1 (loose plant architecture1) in mutant lpa1 controls the leaf lamina joint curvature by inhibiting the auxin signaling associated with C-22-hydroxylation and 6-deoxy-brassinolide, resulting in leaf angle inclination [23].

Panicle neck length (PNL) in the female parent is involved in the yield of hybrid rice seed production by affecting the reception of pollens. The panicles of cytoplasmic male sterile plants are partially or fully enclosed in the flag leaf sheath due to the reduction of indigenous GAs[24]. Rutger and Carnahan [25] discovered an elongated uppermost internode (eui) mutant in a japonica rice population, and elucidated that the elongated uppermost internode trait was controlled by recessive alleles at the eui locus. To date, at least 54 QTLs for the panicle neck length have been reported using bi-parental segregating populations. The number of QTLs on chromosome 1 to 12 is 9, 5, 6, 2, 5, 2, 5, 7, 1, 4, 4, and 4, respectively [7,26–33].

A chromosome segment substitution line (CSSL) population constructed by multiple continuous backcrossing combined with molecular marker assisted selection can be efficiently utilized for QTL mapping [34–36] because each line of the CSSL population usually contains one or more segments from the donor parent. Wang et al. (2006) proposed a method of the likelihood ratio test based on stepwise regression (RSTEP-LRT) that can be used for QTL mapping in a population consisting of non-idealized CSS lines (idealized CSS line means that each line in the CSSL population contains only one segment from the donor parent). This method is equivalent to the standard t-test used for idealized CSS lines to select the most important segment for the trait of interest [37].
The objectives of this study were to construct a CSSL population using a japonica restorer C-bao as the receptor parent and a primitive japonica accession Ludao as the donor parent, and to detect QTLs for the four outcrossing traits in rice by deploying the RSTEP-LRT method.

2. Materials and Methods

2.1. Plant Materials and Field Planting

To construct a CSSL population with C-bao as the receptor parent and Ludao as the donor parent (the population was named the CL-CSSL population, C stands for C-bao, and L represents Ludao), we planted the two parents in the paddy field of Jiangpu Experimental Station (118°37′ E, 32°02′ N), Nanjing Agricultural University, Nanjing, China. The corresponding author obtained the Ludao germplasm from Professor Linghua Tang, a rice scientist in the Laboratory of Resources, Food Crop Research Institute, Jiangsu Academy of Agricultural Sciences, Nanjing, China [38]. Ludao is a primitive japonica rice of O. sativa L., which remained an allele of common wild rice (O. rufipogon Griff) [39], and grows naturally in the field of Lianyungang region (lower Yangtze valley), east China’s Jiangsu province [40]. It has strong vigor and unique features favorable to outcrossing, such as a wider flag leaf angle and elongated panicle neck (Figure 1A). C-bao is the restorer line of the japonica hybrid rice cultivar ‘Dang You C-bao’ [41]. Restorer C-bao was bred from the progenies of the combination C57 × Chengbao 1hao, using the pedigree method combined with the restoring ability test process in Anhui Academy of Agricultural Sciences, Hefei, China. C57, the earliest and famous japonica rice restorer line bred by Professor Yang et al. [42]), was bred from a multiple cross combination ([IR8 × Keqing 3hao) F1 × Jingyin 35]. All these parents used for breeding C-bao are japonica types except the restoring genes derived from an indica cultivar IR8.

![Figure 1](image-url)

Figure 1. The plants, flag leaf blades, and panicles of parents, and partial lines in the population of chromosome segment substitution line with C bao as a receptor parent and Ludao as a donor parent (CL-CSSL) growing in the paddy rice field. (A) Plants of Ludao (left) and C-bao (right); (B) Flag leaf blade length and width; (C) Flag leaf angle; (D) Panicle neck length; (E) Partial lines of the CL-CSSL population, showing a difference between two parents and variation among lines in the CL-CSSL population.

The construction of the CL-CSSL population began in 2004. From 2004 to 2016, the plant materials of parents and segregating populations were planted in the paddy field of Jiangpu Experimental Station. The procedure of the CL-CSSL population construction included hybridization of two parents, C-bao as a pollen parent backcrossing to the F1 pants, selfing the BC1F1 plants 3 generations to BC1F4, multiple continuous backcrossing to the BC1F4 progeny population plants by C-bao, and at BC3F5 generation, SSR markers were used to genotype the 124 introgressive lines for screening lines of interested, selfing seeds of the interested lines were
harvested and sown next year, and new segregants from the progeny of plants with heterozygous marker locus were selected by SSR marker dissection. At last, 163 CSS lines from the BC3F6 generation were obtained and formed the CL-CSSL population (Figure 2).

**Figure 2.** The breeding procedure of the CL-CSSL population used.

For QTL detection, the CL-CSSL population comprising 163 introgression lines and their two parents were planted in 2017 and 2018 using a randomized complete block design (RCBD) with four replications at Jiangpu Experimental Station. In 2017, seeds were sown on a nursery bed of paddy fields on 12 May, and the 30-day-old seedlings were transplanted with one plant per hill. Each line was planted in two rows with eight hills per row. The density was 17 cm × 20 cm. Standard field management practices were followed. In 2018, the same procedure was followed as mentioned for 2017.

### 2.2. DNA Extraction and SSR Marker Genotyping

The genomic DNA was extracted from the young leaves of Ludao, C-bao, and the 163 lines in the CL-CSSL population at the tillering stage, using the method described by Murray and Thompson [43]. According to the rice molecular linkage map and microsatellite database published by Temnykh et al. [44] and McCouch et al. [45], 830 simple sequence repeats (SSR) primers were chosen for detecting the polymorphism between the two parents. The 830 primers were synthesized by Shanghai Generay Biotech Co. Ltd., Shanghai, China. After electrophoresis, 97 SSR primers showed polymorphism between the two parents and were used to genotype the 163 lines. A total volume of 10 μL PCR reaction mixture containing 20 ng genomic DNA, 0.14 pM of each forward and reverse primer, 0.1% Triton X-100, 10 mM Tris HCl (pH 9.0), 0.5 mM dNTPs, 0.5 U of Taq polymerase, and 1.5 mM MgCl2 was used. The amplification was done in a PTC-100 PCR machine (MJ Research™, Ramsey, MN, USA) programmed as 95 °C for 5 min, followed by 28 cycles for 30 s at
95 °C, 30 s at 50 °C to 65 °C (based on primers), extension for 1 min at 72 °C, and a final extension at 72 °C for 7 min and a 4 °C hold. The amplified products were electrophoresed on an 8.0% polyacrylamide gel at 180 electric voltage with a constant electric current and visualized using silver staining. Fragment length was estimated by comparison with standard size markers (100 bp ladder) and recorded.

2.3. Phenotyping CL-CSSL Population and Their Parents

After 25 days of heading, three plants in the middle row of each plot from each replication were randomly selected to measure the flag leaf length (FLL; in cm, Figure 1B), flag leaf width (FLW; in cm, Figure 1B), flag leaf angle (FLA; in degree (°) measured with a protractor, Figure 1C), and panicle neck length (PNL; in cm, from the uppermost node to the panicle neck node (Figure 1D). The average values of three measurements for each replicate was used for further analysis.

2.4. Calculation of Heritability in Broad Sense

Heritability in the broad sense ($H^2$) describes the percentage of phenotypic variation caused by genetic factors. It was calculated based on analysis of variance for the CL-CSSL population using the following formula as described by Dang et al. [46]:

$$H^2 = \sigma^2_g / (\sigma^2_g + \sigma^2_e)n,$$

where $\sigma^2_g$ represents the genetic variance, $\sigma^2_e$ signifies the error variance, and $n$ shows the replicate number.

2.5. Detection of QTLs for the FLL, FLW, FLA, and PNL Traits by the RSTEP-LRT Method

Phenotypic data of the outcrossing traits were analyzed by using the SAS statistical package version 8 (SAS Institute Inc., Cary, NC, USA, http://sas.com/en_us/home.html). Since the majority lines of the CSSL population contained more than one segment from the donor parent, the method of the likelihood ratio test based on stepwise regression (RSTEP-LRT) proposed by Wang et al. [37] was used for QTL mapping in the non-idealized CSS lines, which is equivalent to the standard $t$-test for idealized CSS lines. The linear model of the RSTEP-LRT method was as follows:

$$y_i = b_0 + \sum_{j=1}^l b_j x_{ij} + e_i,$$

where $i = 0$ (for the receptor parent), 1, 2, ..., $n$, $b_0$ is the intercept, and $b_j$ ($j = 1, ..., l$) is the partial regression coefficient of phenotype on the $j$th marker, which indicates an additive effect of QTL on each segment. $x_{ij}$ is the $j$th marker variable in the $i$th CSS line, and $e_i$ is the random experimental error. QTLs for the trait of interest were detected by deploying an RSTEP-LRT method in ICI mapping software Version 4.0 [47]. The QTL nomenclature follows the principles reported by McCouch [48].

3. Results

3.1. Construction of the CL-CSSL Population and Its Characteristics of Composition

Among the 830 pairs of SSR primers screened, 97 pairs of SSR primers were found as polymorphic between C-bao and Ludao, showing 11.8% of the polymorphism rate between the parents (Table 1).

The 97 markers (polymorphic primers) were distributed on all 12 chromosomes of rice. Chromosome 2 showed the highest number of SSR markers (13), and chromosome 12 showed the lowest number of SSR markers (4) (Supplementary Table S1).
Table 1. Distribution of tested and polymorphic SSR markers on 12 chromosomes of rice.

| Chromosome | Number of SSR Primers Tested on Parents | Number of Markers | Polymorphic Rate% |
|------------|-----------------------------------------|-------------------|-------------------|
| 1          | 74                                      | 12                | 16.2              |
| 2          | 78                                      | 13                | 16.7              |
| 3          | 69                                      | 5                 | 7.2               |
| 4          | 73                                      | 7                 | 9.6               |
| 5          | 75                                      | 7                 | 9.3               |
| 6          | 66                                      | 8                 | 12.1              |
| 7          | 62                                      | 9                 | 14.5              |
| 8          | 60                                      | 8                 | 13.3              |
| 9          | 76                                      | 7                 | 9.2               |
| 10         | 62                                      | 7                 | 11.3              |
| 11         | 62                                      | 10                | 16.1              |
| 12         | 74                                      | 4                 | 5.4               |
| Total      | 830                                     | 97                | 11.8              |

The distribution of donor segments on chromosomes in each line of the CL-CSSL population is shown in Supplementary Table S2. The constructed CL-CSSL population covered 90.71% of the genome of the donor parent, Ludao. The graphical genotypes of the 163 CSS lines constructed using the method described by Young et al. [49] are shown in Figure 3.

![Graphical genotypes of 163 CSSLs with C-bao as a receptor parent and Ludao as a donor parent in rice. Note: Blue, white, and red represents the genotype of Ludao, C-bao, and missing band, respectively.](image)
Among the 163 CL-CSSL lines, the number of donor segments ranged from 1 (CSSL075, CSSL076, and CSSL150) to 18 (CSSL049), averaging 8.02 (Figure 4; Supplementary Tables S2 and S3).

![Figure 4](image)

**Figure 4.** Frequency distribution of donor segments from Ludao in the CL-CSSL population.

### 3.2. Phenotypic Variation of the Four Outcrossing Traits in the CL-CSSL Population

Descriptive statistics between two parents and CSSLs population related to the four outcrossing traits are listed in Table 2. The frequency distribution of the four traits in the CL-CSSL population was close to normal (Figure 5).
Figure 5. Frequency distribution of the four outcrossing traits in the CL-CSSL population in 2017 (A–D) and in 2018 (E–H). FLL, flag leaf length; FLW, flag leaf width; FLA, flag leaf angle; PNL, panicle neck length. C stands for the parent of ‘C-bao’, and L for ‘Ludao’.
Larger FLL were observed in Ludao, with a mean value of 50.6 cm, while in C-bao the average FLL was 30.5 cm in 2017. In the CL-CSSL population, the mean value for FLL was 43.9 cm, ranging from 31.1 to 57.8 cm, with a coefficient of variation (CV) of 14.1% in 2017. Similar results were observed in 2018 (Table 2).

The mean value for the flag leaf width in Ludao was 1.5 cm whereas in C-bao it was 1.5 cm in 2017. In 2017, the mean FLW over the 163 CSSLs was 1.7 cm, ranging from 1.4 to 2.1 cm, with a CV of 5.9%. In 2018, similar results were observed for FLW (Table 2).

For FLA, Ludao had a mean value of 115.0° and C-bao had 22.9° in 2017. The average value of FLA for the CSSL population was 18.8°, ranging from 5.0° to 60.8°, with a 39.9% of CV in 2017. In 2018, similar results were noticed (Table 2).

Significantly longer panicle neck lengths were recorded in Ludao, with a mean value of 6.4 cm, while C-bao had a mean value of 0.0 cm in 2017. In the CL-CSSL population, the PNL had a mean value of 5.1 cm, ranging from 0.0 to 12.0 cm, with a 60.8% of CV in 2017. Similar results were observed for PNL in 2018 (Table 2). Based on the data of heritabilities of the 4 traits calculated across two years, it could be concluded that the performance of the four outcrossing-related traits were stable during the two years of investigation, hence they were mainly controlled by genetic factors (Table 2).

| Year | Trait | Ludao (Mean ± SD) | C-bao (Mean ± SD) | Difference Between Parents | CL-CSSL Population (Mean ± SD) | CV/IFB |
|------|-------|-------------------|-------------------|---------------------------|-------------------------------|-------|
| 2017 | FLL, cm | 50.6 ± 1.2 | 30.5 ± 0.7 | 20.1 *** | 43.9 ± 6.2 | 31.1-57.8 | 14.1 | 94.0 |
|      | FLW, cm | 1.5 ± 0.1 | 1.5 ± 0.1 | 0.0 ** | 1.7 ± 0.1 | 1.4-2.1 | 5.9 | 99.0 |
|      | FLA, ° | 115.0 ± 2.7 | 22.9 ± 2.5 | 92.1 *** | 18.8 ± 7.5 | 5.0-60.8 | 39.9 | 98.4 |
|      | PNL, cm | 6.4 ± 0.7 | 0.0 ± 0.0 | 6.4 *** | 5.1 ± 3.1 | 0.0-12.0 | 60.8 | 99.5 |
| 2018 | FLL, cm | 52.1 ± 1.7 | 31.3 ± 1.1 | 20.8 *** | 41.8 ± 4.7 | 28.9-63.0 | 11.2 | 91.0 |
|      | FLW, cm | 1.3 ± 0.3 | 1.5 ± 0.1 | −0.2 * | 1.7 ± 0.1 | 1.2-2.5 | 5.9 | 96.8 |
|      | FLA, ° | 115.9 ± 2.7 | 18.7 ± 5.2 | 97.2 *** | 20.7 ± 8.8 | 5.0-65.0 | 42.5 | 98.8 |
|      | PNL, cm | 5.5 ± 1.7 | 0.0 ± 0.0 | 5.5 ** | 5.3 ± 3.4 | 0.0-16.5 | 64.1 | 98.2 |

FLL, Flag leaf length; FLW, Flag leaf width; FLA, Flag leaf angle; PNL, Panicle neck length. *, **, and *** represent significance at α = 0.05, α = 0.01, and α = 0.001 probability level, respectively, by t-test.

The correlation coefficients (r) among the four phenotypic traits ranged from −0.36 to 0.54 (Table 3). The highest significant positive correlation was observed between FLA and PNL (r = 0.45, p < 0.001 in 2017 and r = 0.54, p < 0.001 in 2018) and the highest significant negative correlation was noticed between PNL and FLW (r = −0.36, p < 0.001 in 2017 and r = −0.25, p < 0.001 in 2018). These results indicated that the lines with longer PNL also showed larger FLA and narrower FLW, which is beneficial for improving the three outcrossing traits simultaneously, but having longer FLL, which is not desired to improve the two traits at the same time. Therefore, it is necessary to mine favorable alleles for each trait separately and then recombine them together to improve the two traits.

| Year | FLL | FLW | FLA | PNL |
|------|-----|-----|-----|-----|
| 2017 | 1   |     |     |     |
| 2018 | 1   |     |     |     |
| 2017 | 0.07 | 1   |     |     |
| 2018 | 0.22 ** | 1   |     |     |
| 2017 | −0.02 | −0.25 ** | 1   |
3.3. Detection of QTLs for FLL, FLW, FLA, and PNL Traits in the CL-CSSL Population

Seven QTLs for the four outcrossing traits were detected through the RSTEP-LRT method under the LOD threshold of 2.5. The percentage of phenotypic variance explained (PVE) by each QTL ranged from 6.49% (linked to RM349) to 21.33% (linked to RM7075). Among the seven QTLs detected, four QTLs were repeatedly detected in both years (Table 4).

3.3.1. QTL for Flag Leaf Length

One QTL, qFLL-4 linked with SSR marker locus RM349 detected on chromosome 4, explained 6.70% of the two-year-averaged phenotypic variance of the FLL, and the alleles from Ludao decreased FLL by 5.1 cm (Table 4).

3.3.2. QTL for Flag Leaf Width

One QTL (qFLW-11) linked with RM286 was detected on chromosome 11 with a contribution rate of 13.91%, and the allele from C-bao parent decreased 0.12 cm of the flag leaf width (Table 4).

3.3.3. QTLs for Flag Leaf Angle

Three QTLs for the flag leaf angle (qFLA-1.1, qFLA-1.2, and qFLA-10) were detected across two environments. qFLA-1.1 and qFLA-1.2 explained 7.85% and 21.29% of the 2-year-averaged phenotypic variance, respectively, and the alleles from Ludao increased FLA 17.38°and 31.50°. The qFLA-10 linked with RM1125 detected on chromosome 10 in 2018 only showed PVE 7.74% and the allele from Ludao had an increasing effect of 9.77° on the flag leaf angle (Table 4).

3.3.4. QTLs for Panicle Neck Length

Two QTLs were detected for the panicle neck length. qPNL-7 linked with RM11 detected on chromosome 7 showed PVE 7.07%, and the allele from Ludao parent had decreasing effect of 1.23 cm on PNL in 2017. qPNL-8 linked with RM72 detected on chromosome 8 showed a contribution rate of 8.76% and the allele from Ludao had an increasing effect of 2.04 cm in 2017 and 8.99% of PVE with a 2.40 cm increasing effect in 2018. Averaged over 2 years, qPNL-8 explained 8.87% of the phenotypic variance, and the allele from Ludao increased PNL 2.22 cm.

| Locus | Marker Name | Chr. | 2017 LOD Value | PVE (%) | Add | 2018 LOD Value | PVE (%) | Add |
|-------|-------------|------|----------------|---------|-----|----------------|---------|-----|
| qFLL-4 | RM349       | 4    | 2.5            | 6.49    | -2.55 | 2.53           | 6.92    | -2.5 |
| qFLW-11 | RM286     | 11   | 5.33           | 13.91   | 0.12 | -              | -       | -   |
| qFLA-1.1 | RM8105  | 1    | 3.67           | 7.87    | 8.71 | 3.65           | 7.84    | 8.67 |
| qFLA-1.2 | RM7075   | 1    | 8.92           | 21.33   | 15.78 | 8.88           | 21.26   | 15.72 |
| qFLA-10 | RM1125     | 10   | -              | -       | -    | 2.87           | 7.74    | 9.77 |
| qPNL-7 | RM11       | 7    | 2.61           | 7.07    | -1.23 | -              | -       | -   |
| qPNL-8 | RM72       | 8    | 3.26           | 8.76    | 2.04 | 3.36           | 8.99    | 2.4  |

Chr. Chromosome; PVE percentage of phenotypic variance explained; Add additive effect.
4. Discussion

In this study, we detected seven QTLs for the four outcrossing-related traits by the RSTEP-LRT method using ICI mapping software under the LOD threshold value of 2.5. Among them, four QTLs (qFLL-4, qFLA-1.1, qFLA-1.2, and qPNL-8) were detected in both years. In comparison with the results obtained by previous researchers, although some QTLs for FLL (linked with RM1112, RM1272, RM6314, RM6997, RM6114, and RM3843) have also been detected on chromosome 4 [6,7,50–54], QTL qFLL-4 linked with RM349 on chromosome 4 detected in our research was a new one, explaining 6.7% of the two-year-averaged phenotypic variance, and the allele from Ludao decreased FLL 5.10 cm if the chromosome region of the locus in restorer C-bao was substituted by Ludao (Table 4). Around the region of RM349, two agronomically important genes have been cloned. One is Bph3, which controls brown plant hopper resistance [55]. The other is the sh4 gene, which confers seed shattering in rice [56]. Ludao is a primitive japonica rice accession and the ripe seed is almost completely fallen to the ground by shattering before harvest. We speculated that FLL may also be related with brown plant hopper resistance and seed shattering.

For FLW, the allele from Ludao on QTL qFLW-11 could decrease flag leaf width by 0.24 cm (Table 4) and could be used in practice for breeding maintainer lines with a narrow flag leaf width, although the locus was detected in one year.

The broad-sense heritability of FLA exceeded 95% in both years of investigation, which means that the variation of the FLA is controlled mainly by the genetic factor and less affected by the environmental factors. Three QTLs, qFLA-1.1, qFLA-1.2, and qFLA-10 linked with RM6105 on chromosome 1, RM7075 on chromosome 1, and RM1125 on chromosome 10, respectively, were detected for FLA, with the PVE ranging from 7.74% to 21.33%. Among these detected QTLs, qFLA-1.2 showed the highest two-year-averaged PVE of 21.29% and the favorable alleles were from the Ludao parent. The flag leaf angle increased 31.50° if chromosome regions of this locus in C-bao were substituted by Ludao. A database search revealed that a putative gene encoding an RNA-dependent RNA polymerase (RdRP) was located in the same region, which was involved in the trans-acting small interfering RNA (ta-siRNA) pathway [57]. Genes involved in the ta-siRNA pathway, such as shoot organization (SHO1 and SHO2) in rice, are responsible for the regulation of adaxial-axial polarity in the leaf [58]. We speculated that the QTL qFLA-1.2 detected in our study might be related to the adaxial-axial polarity in the leaf, and this locus is worthy of further study.

For PNL, two QTLs (qPNL-7, qPNL-8) were detected as being linked with RM11 on chromosome 7 and RM72 on chromosome 8. qPNL-8 was detected in both years, with two-year-averaged PVE of 8.87%. Alleles from Ludao increased the panicle neck length by 5.56 cm and could be used to improve the PNL of CMS lines (first maintainers). Since a positive correlation between PNL and FLL was observed (Table 3), the longer PNL will also lead to a longer FLL, which is not desired for hybrid seed production. Fortunately, both traits were controlled by multiple QTLs, and both positive and negative alleles existed among the parents. We can introduce the favorable allele of qFLL-4 and favorable allele of qPNL-8 from Ludao (Table 4) into a superior female parent to improve PNL and FLL simultaneously for hybrid rice seed production.

We screened 830 SSR primers and obtained 97 primers showing polymorphic markers between two parents (polymorphic rate 11.8%), confirming that Ludao belongs to primitive japonica types and has a close relationship with japonica cultivars. The low number of polymorphic markers might lead to a biased QTL location or overestimated QTL effect, although the CSSL population was used.

5. Conclusions

Four new QTLs for FLL, FLA, and PNL were detected in both years in the CL-CSSL population, and the alleles from the donor parent Ludao can be used for breeding a CMS line with a shorter FLL, wider FLA, and longer PNL, which is beneficial for hybrid rice seed production.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Summary information of SSR markers in CL-CSSL population, Table S2: Distribution of donor segments on each line and
chromosome in CL-CSSL population, Table S3: Percentage of donor marker segments of 163 lines in the CL-CSSL population

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