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ARTICLE

Multi-Environmental Genetic Analysis of Grain Size Traits Based on Chromosome Segment Substitution Line in Rice (*Oryza sativa* L.)

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ABSTRACT

Grain size traits are critical agronomic traits which directly determine grain yield, but the genetic bases of these traits are still not well understood. In this study, a total of 154 chromosome segment substitution lines (CSSLs) population derived from a cross between a *japonica* variety Koshihikari and an *indica* variety Nona Bokra was used to investigate grain length (GL), grain width (GW), length-width ratio (LWR), grain perimeter (GP), grain area (GA), and thousand grain weight (TGW) under four environments. QTL mapping analysis of six grain size traits was performed by QTL IciMapping 4.2 with an inclusive composite interval mapping (ICIM) model. A total of 64 QTLs were identified for these traits, which mapped to chromosomes 1, 2, 3, 4, 6, 7, 8, 10, 11, and 12 and accounted for 1.6%–27.1% of the total phenotypic variations. Among these QTLs, thirty-six loci were novel and seven QTLs were identified under four environments. One locus containing the known grain size gene, *qGL3*/*GL3.1*/*OsPPKL1*, also have been found. Moreover, five pairs of digenic epistatic interactions were identified except for GL and GP. These findings will facilitate fine mapping of the candidate gene and QTL pyramiding to genetically improve grain yield in rice.

KEYWORDS

Rice; grain size; quantitative trait loci; epistatic; chromosome segment substitution lines

1 Introduction

Rice (*Oryza sativa* L.) is an important staple food feeding more than half of the world's population. To meet the demand for food for the rapid growth of the population, continuous improvements of the rice yield will be necessary [1]. Rice grain yield is major determined by the number of panicles per plant or effective tillers per plant, the number of grains per panicle, and the grain weight [2,3]. Grain size, which is closely

associated with grain weight, is made up of grain length, grain width, grain thickness, and length-to-width ratio, etc., which are key determinants of grain yield and grain quality [4,5]. Therefore, the improvement of grain size traits is one of the most important tasks for rice breeders to increase rice production.

Rice grain size traits are genetically controlled by numerous quantitative trait loci (QTLs) [6]. To date, QTL mapping for rice grain size traits using different populations, which derived from various biparental crosses, has been reported in many previous studies [7–17]. Some of QTLs identified in these populations have been cloned, such as *GS3* [18], *GW2* [19], *GW5*/*qSW5*/*GSE5* [20–23], *GS5* [24], *qGL3*/*GL3.1*/*OsPPKL1* [25–27], *GW8*/*OsSPL16* [28], *TGW6* [29], *GW6a* [30], *GW7*/*GL7*/*SLG7* [31–33], *GS2*/*GL2*/*GLW2*/*PT2* [34–38], *GLW7* [39], *GS9* [40], *qLGY3*/*OsLG3b* [41,42], *TGW3*/*qTGW3*/*GL3.3*/*OsSK41* [43–45], *TGW2* [46], *OsAUX3* [47]. Although a large number of QTLs for grain size traits have been mapped or cloned, and these results have facilitated a better understanding of the genetic basis of the grain size, it still needs to identify new QTL or genes to understand the complex mechanisms of grain size traits.

Using chromosome segment substitution lines (CSSLs) is a good strategy for QTL mapping. The substitution lines are the complete integration of the substituted segments with the stability of a certain character, which could reduce the interference of background noise and improve the accuracy of QTL mapping [48]. A large number of CSSLs sets have been developed to identify the QTLs for rice agronomic traits, including the grain size traits [47,49–53].

Koshihikari is a well-known *japonica* rice variety in Japan, which exhibits a good eating quality, cold tolerance at the booting stage, and high resistance to pre-harvest germination [54]. Nona Bokra is a typical *indica* variety from India with high tolerance to salt stress [55,56]. Utilization of Koshihikari and Nona Bokra to identify QTLs for salt tolerance and rice grain quality have been reported [54,55], but there is no report on grain size traits. In this study, a CSSLs population containing 154 lines, which is derived from a cross between Koshihikari and Nona Bokra, was used to analyze the QTLs for grain size traits in rice under four environments. The results could provide valuable information to further elucidate the genetic basis of rice grain size as well as for marker-assisted selection in rice breeding.

2 Materials and Methods

2.1 Plant Materials

The CSSLs population consisting of 154 lines was derived from the cross of a *japonica* cultivar Koshihikari (recipient parent) and an *indica* rice cultivar Nona Bokra (donor parent). The construction of the CSSLs population was described by Hao et al. [54]. In brief, the F_1 plants were obtained from the cross of Koshihikari and Nona Bokra, and then backcrossed to Nona Bokra three times to produce 680 BC_3F_1 seed. Based on 102 polymorphisms CAPS and SSR markers covering the whole rice genome, 71 individuals which have a single, relatively long heterozygous chromosome segment were selected [57,58]. a total of 3266 BC_3F_2 plants were produced from the self-pollinated of 71 individuals. Based on the marker-assisted selection (MAS), 154 BC_3F_2 plants were finally selected as the CSSLs population. The field trials were conducted in Shanghai (SH, 121°24′ E, 31°00′ N) and Lingshui (LS, 110°00′ E, 18°31′ N) during the 2015 and 2016 rice-growing seasons, respectively. Trials in each environment were laid out in a completely randomized block design, with a planting density of 20 cm × 20 cm. Three replicates were growth and each line was planted in four rows with six plants. The management of field experiments including irrigation, fertilizer application and pest control, was in line with normal agricultural standard practices.

2.2 Evaluation of Grain Size Related Traits

Approximately 40 days after flowering, rice grains were harvested. Freshly harvested paddy was air-dried and stored at room temperature for 3 months before testing. Six grain size traits were measured in this study, such as grain length (GL), grain width (GW), length-width ratio (LWR), grain perimeter (GP),

grain area (GA), and thousand grain weight (TGW). The GL, GW, GP, GA, and TGW traits were evaluated using WSeen SC-G automatic seed testing system and thousand grain weight analysis system (Hangzhou WSeen Detection Technology Co., Ltd., China). About 100–200 grains for each entry were evaluated. LWR was calculated as the ratio of GL and GW.

2.3 Data Analysis

The phenotypic data of the two parents and the CSSLs population was analyzed using Microsoft Excel 2016. The density plot was analyzed using the ggplot2 package in R. Trait correlations were calculated and plotted using the corrplot package in R.

2.4 Identification of QTLs

A total of 126 polymorphism markers between Koshihikari and Nona Bokra was selected to screen the CSSLs population. QTLs for each trait were detected using QTL IciMapping 4.2 with inclusive composite interval mapping (ICIM) model, and a threshold LOD of 2.5 was applied [59,60]. The additive effects, genetic parameters and variation percentages of the QTLs were also estimated. The QTL named followed the method of McCouch et al. [61]. The genetic map was drawn using MapChart version 2.32 software [62].

2.5 DNA Extraction and Gene Sequencing

Genomic DNA was extracted from fresh leaves using CTAB method [63]. The coding region of *qGL3/GL3.1/OsPPKL1* was amplified from genomic DNA using 2 × Phanta Max Master Mix (Vazyme, Nanjing, China). PCRs were conducted using standard PCR protocols. The primers used for sequencing are listed in Table S1.

3 Results

3.1 Phenotypic Variation in Parents and the CSSLs Population

The grain size traits of the parents and 154 CSSLs population, such as grain length (GL), grain width (GW), length-width ratio (LWR), grain perimeter (GP), grain area (GA), and thousand grain weight (TGW), were list in Table 1. Because of the insensitivity to photoperiod, the *indica* variety Nona Bokra could not harvest the seeds in Shanghai. Therefore, we can only compare the grain size traits of the parental in Lingshui. All traits exhibited significant differences between parents in Lingshui 2015 and 2016. The value of grain size traits in Nona

Table 1: Description of grain size traits of two parents and their CSSLs population in four environments

Year and location	Traits	Parents (Mean ± SD)			CSSLs population			
		Koshihikari	Nona Bokra	*p* value	Mean ± SD	Range	Skewness	Kurtosis
2015 Shanghai	GL (mm)	7.49	—	—	7.47 ± 0.22	6.87~8.11	0.23	0.94
	GW (mm)	3.42	—	—	3.39 ± 0.12	3.11~3.70	0.08	−0.17
	LWR	2.20	—	—	2.22 ± 0.09	2.00~2.50	0.24	0.58
	GP (mm)	18.87	—	—	18.69 ± 0.50	17.29~20.27	0.12	0.96
	GA (mm^2)	18.96	—	—	18.57 ± 0.94	16.33~21.90	0.26	0.69
	TGW (g)	25.94	—	—	24.44 ± 1.99	18.33~29.38	−0.16	0.13
2015 Lingshui	GL (mm)	7.06	7.97	<0.0001	7.04 ± 0.22	6.53~7.72	0.57	0.49
	GW (mm)	3.30	3.13	0.0042	3.29 ± 0.11	2.96~3.61	−0.23	0.27
	LWR	2.15	2.56	<0.0001	2.16 ± 0.09	1.96~2.48	0.76	1.00
	GP (mm)	17.76	19.45	<0.0001	17.76 ± 0.49	16.72~19.38	0.37	0.22
	GA (mm^2)	17.08	18.59	0.0034	16.84 ± 0.84	14.87~19.48	0.25	0.32
	TGW (g)	22.60	26.98	<0.0001	22.26 ± 1.62	18.30~26.08	−0.10	−0.40

(Continued)

Table 1 (continued)

Year and location	Traits	Parents (Mean ± SD)			CSSLs population			
		Koshihikari	Nona Bokra	*p* value	Mean ± SD	Range	Skewness	Kurtosis
2016 Shanghai	GL (mm)	7.63	—	—	7.59 ± 0.22	7.04~8.24	0.61	0.63
	GW (mm)	3.40	—	—	3.33 ± 0.12	3.07~3.62	−0.02	−0.09
	LWR	2.25	—	—	2.29 ± 0.09	2.07~2.52	0.24	−0.10
	GP (mm)	19.16	—	—	19.01 ± 0.49	17.71~20.60	0.49	0.69
	GA (mm^2)	18.76	—	—	18.27 ± 0.89	16.52~20.89	0.42	−0.04
	TGW (g)	24.46	—	—	23.13 ± 1.74	18.87~29.20	0.08	0.61
2016 Lingshui	GL (mm)	7.12	8.46	<0.0001	7.19 ± 0.21	6.75~7.76	0.57	0.21
	GW (mm)	3.30	3.23	0.0012	3.34 ± 0.10	3.08~3.63	−0.02	0.13
	LWR	2.17	2.63	<0.0001	2.17 ± 0.09	1.98~2.44	0.52	0.60
	GP (mm)	17.99	20.55	<0.0001	18.12 ± 0.45	17.03~19.28	0.33	−0.10
	GA (mm^2)	17.51	20.39	<0.0001	17.72 ± 0.76	16.05~19.69	0.26	−0.08
	TGW (g)	25.00	28.52	0.0076	24.33 ± 1.62	19.56~29.02	−0.09	0.12

Note: GL: Grain length; GW: Grain width; LWR: Length width ratio; GP: Grain perimeter; GA: Grain area; TGW: Thousand grain weight.

Bokra was significantly higher than that of Koshihikari except for GW in Lingshui 2015 and 2016. A wide range of values for all the grain size traits was observed in CSSLs population (Table 1). The frequency distribution of each trait displayed a continuous variation under four environments, and the absolute values of skewness and kurtosis were less than 1, suggesting these traits followed a normal distribution and were suitable for QTL analysis (Fig. 1, Table 1).

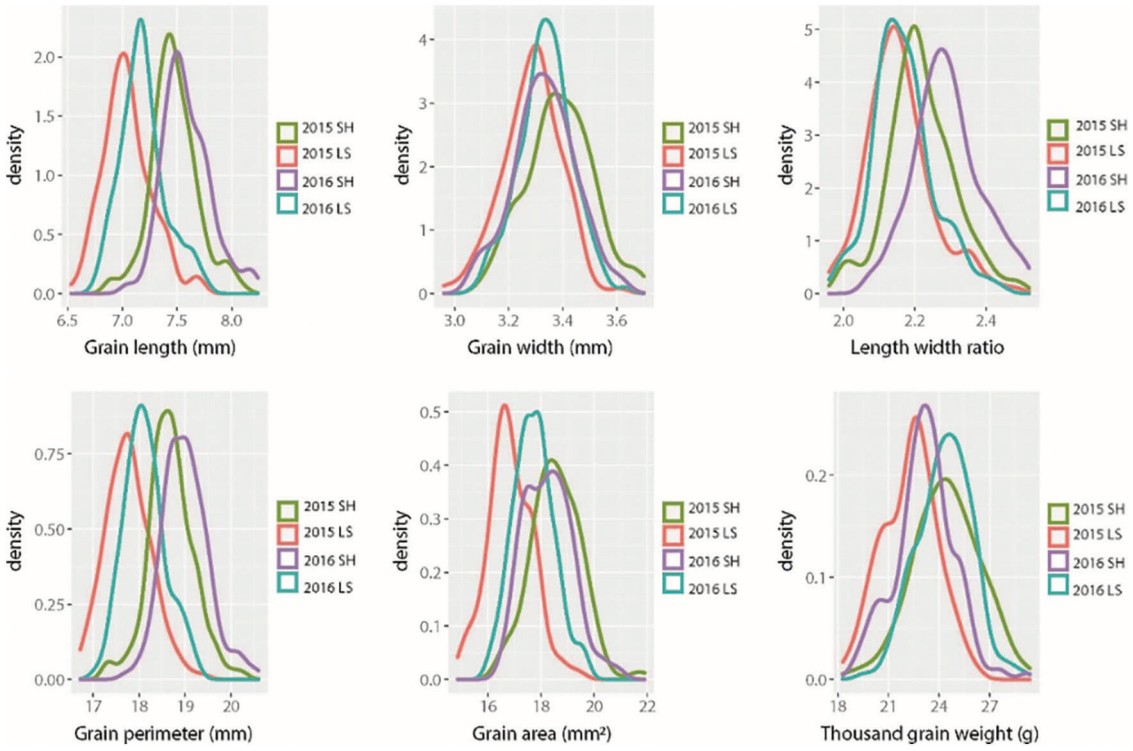

Figure 1: Frequency distributions of six grain size traits in CSSLs population in four environments. SH: Shanghai; LS: Lingshui

3.2 Correlation Analysis of Grain Size Traits

The correlation analyses of GW, GL, LWR, GP, GA, and TGW under four environments were performed as shown in Fig. 2. GL and GP showed positive correlations with the other five traits. GL exhibited the highest significant positive correlation with GP, with correlation coefficients reaching up to 0.95 in Shanghai 2015, 0.95 in Lingshui 2015, 0.96 in Shanghai 2016, and 0.95 in Lingshui 2016, indicating that GL was the most important contributor to GP. Similarly, GP exhibited the highest significant positive correlation with GA, the correlations coefficients were 0.88 in Shanghai 2015, 0.87 in Lingshui 2015, 0.89 in Shanghai 2016, and 0.89 in Lingshui 2016. GW showed the highest significant negative correlation with LWR, the correlations coefficients were −0.74, −0.68, −0.72, and −0.68, respectively.

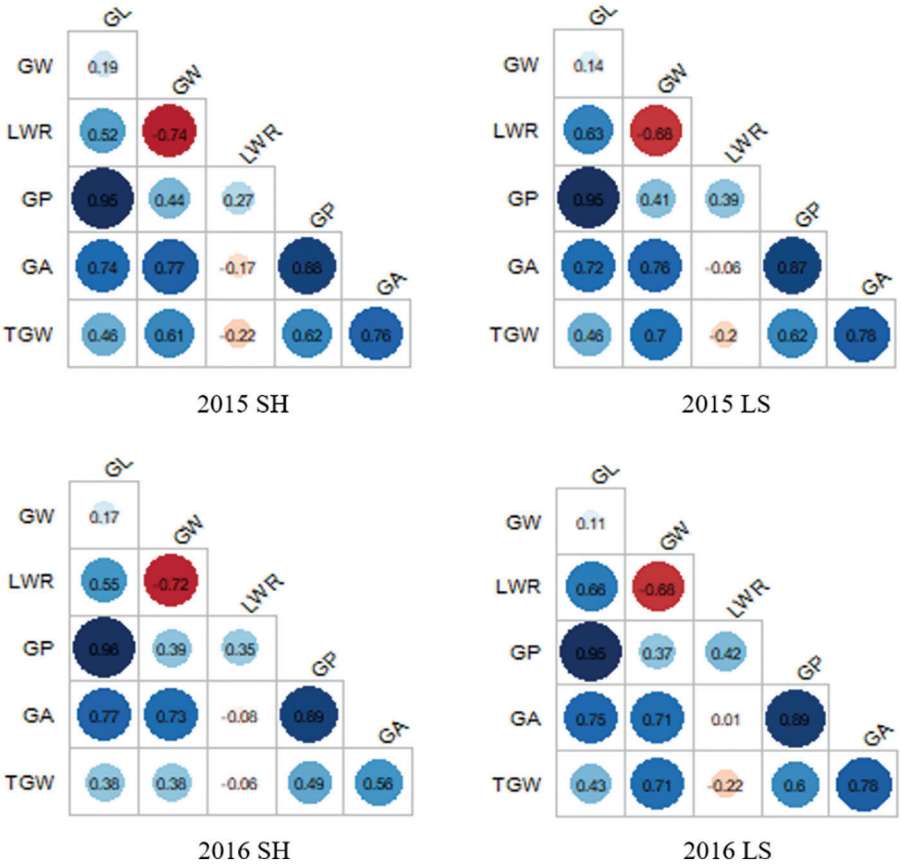

Figure 2: Trait correlations for six grain size traits in CSSLs populations in four environments. SH: Shanghai; LS: Lingshui; GL: Grain length; GW: Grain width; LWR: Length width ratio; GP: Grain perimeter; GA: Grain area; TGW: Thousand grain weight

3.3 Identification QTLs for Grain Size Traits

A total of 64 QTLs were identified for six grain size traits on chromosomes 1, 2, 3, 4, 6, 7, 8, 10, 11, and 12 in Shanghai 2015, Lingshui 2015, Shanghai 2016, and Lingshui 2016. The LOD score values of these QTLs ranged from 2.53 to 29.34, with individually accounted for 1.6% to 27.1% of the phenotypic variation (Fig. 3, Table 2).

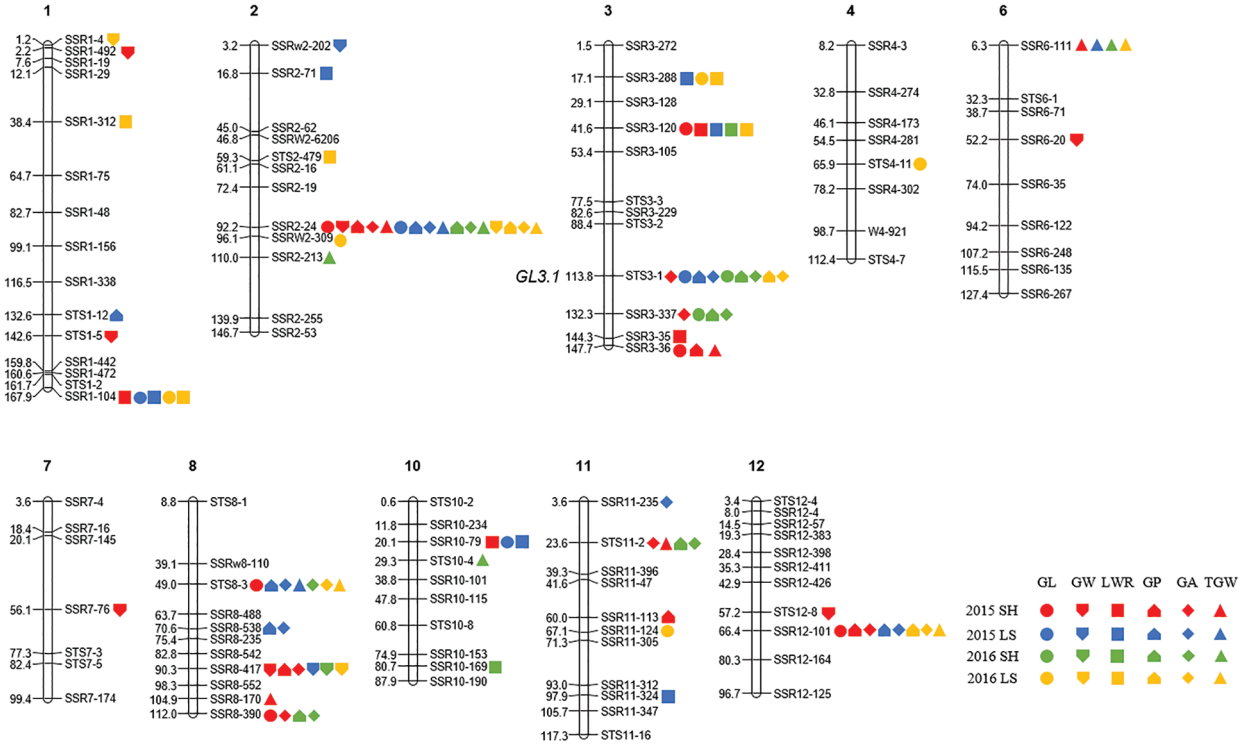

Figure 3: Locations of QTLs for six grain size traits in CSSLs population in four environments. DNA markers are shown on the right side of each chromosome and the genetic distance are shown on the left side of each chromosome. SH: Shanghai; LS: Lingshui; GL: Grain length; GW: Grain width; LWR: Length width ratio; GP: Grain perimeter; GA: Grain area; TGW: Thousand grain weight

For GL, a total of fourteen QTLs, including six in Shanghai 2015, four in Shanghai 2016, two in Lingshui 2015, and five in Lingshui 2016, were detected on chromosomes 1, 2, 3, 4, 8, 10, 11, and 12 (Table 2). *qGL1*, *qGL2-1*, and *qGL3-3* were detected in two environments, respectively, with additive effects ranging from −0.12 to 0.17 and explaining from 7.0% to 12.4% of phenotypic variance. Other eleven QTLs for GL were only detected in one environment, with the explained variation of 4.8% to 11.5%.

A total of nine QTLs were detected on chromosomes 1, 2, 6, 7, 8, and 12 for GW (Table 2). The QTL, *qGW8*, was identified on chromosome 8 under four environments, and the highest phenotypic variation of *qGW8* was detected in Shanghai 2015, reaching 16.5%. Another QTL for GW, *qGW2-2*, was detected in Shanghai 2015 and Lingshui 2016. It explained the variation of 6.3% and 7.8%, respectively. Other 7 QTLs for GW were only detected in one environment, with the explained variation of 3.8% to 12.4%. *qGW2-1* explained the highest phenotypic variation in those 7 QTLs, which reach 12.4%. The allele of *qGW2-1* from Nona Bokra decreased GW by about 0.08% in Lingshui in 2015.

Ten QTLs located on chromosomes 1, 2, 3, 10, and 11 were identified for LWR. *qLWR3-2* was detected under four environments, and explained 7.9%, 9.1%, 11.4%, and 11.6% of the total phenotypic variance under four environments, respectively. The allele of *qLWR3-2* from Nona Bokra increased LWR by about 0.04% in Lingshui in 2015 and 2016. *qLWR1-2* was detected on chromosomes 1 under three environments, and the alleles increasing LWR were contributed by Nona Bokra. *qLWR3-1* was detected in Lingshui 2015, and Lingshui 2016, and *qLWR10-1* was detected in Shanghai 2015 and Lingshui 2015, which explained the variation of 4.1%, 7.1% and 7.1%, 7.8%, respectively. The other six QTLs for LWR were only detected in one environment, and *qLWR3-3* showed the highest phenotypic variation in Shanghai 2015.

Table 2: QTLs for grain size traits in CSSLs population of Koshihikari and Nona Bokra

Traits	QTL	Chr.	Marker name	Position (cM)	LOD				Additive effect				PVE (%)			
					2015SH	2015LS	2016SH	2016LS	2015SH	2015LS	2016SH	2016LS	2015SH	2015LS	2016SH	2016LS
GL	*qGL1*	1	SSR1-104	167.9	—	3.94	—	3.97	—	0.12	—	0.11	—	7.2	—	7.0
	qGL2-1	2	SSR2-24	92.2	4.42	5.70	—	—	−0.09	−0.12	—	—	7.1	10.6	—	—
	qGL2-2	2	SSRW2-309	96.1	—	—	—	5.20	—	—	—	−0.09	—	—	—	9.4
	qGL3-1	3	SSR3-288	17.1	—	—	—	6.24	—	—	—	0.15	—	—	—	11.5
	qGL3-2	3	SSR3-120	41.6	3.06	—	—	—	0.07	—	—	—	4.8	—	—	—
	qGL3-3	3	STS3-1	113.8	—	4.67	8.09	—	—	0.12	0.17	—	—	8.6	12.4	—
	qGL3-4	3	SSR3-337	132.3	—	—	4.60	—	—	—	−0.14	—	—	—	6.7	—
	qGL3-5	3	SSR3-36	147.7	6.45	—	—	—	−0.24	—	—	—	10.7	—	—	—
	qGL4	4	STS4-11	65.9	—	—	—	2.77	—	—	—	0.07	—	—	—	4.8
	qGL8-1	8	STS8-3	49.0	3.89	—	—	—	0.10	—	—	—	6.2	—	—	—
	qGL8-2	8	SSR8-390	112.0	4.39	—	—	—	0.11	—	—	—	7.1	—	—	—
	qGL10	10	SSR10-79	20.1	—	3.00	—	—	—	−0.09	—	—	—	5.4	—	—
	qGL11	11	SSR11-124	67.1	—	—	—	3.81	—	—	—	0.10	—	—	—	6.7
	qGL12	12	SSR12-101	66.4	3.35	—	—	—	−0.10	—	—	—	5.3	—	—	—
GW	*qGW1-1*	1	SSR1-4	1.2	—	—	—	3.14	—	—	—	0.04	—	—	—	6.2
	qGW1-2	1	SSR1-492	2.2	3.74	—	—	—	0.05	—	—	—	4.9	—	—	—
	qGW1-3	1	STS1-5	142.5	3.69	—	—	—	−0.08	—	—	—	4.8	—	—	—
	qGW2-1	2	SSRw2-202	3.2	—	5.58	—	—	—	−0.08	—	—	—	12.4	—	—
	qGW2-2	2	SSR2-24	92.2	4.76	—	—	3.91	−0.05	—	—	-0.04	6.3	—	—	7.8
	qGW6	6	SSR6-20	52.2	3.86	—	—	—	−0.05	—	—	—	5.0	—	—	—
	qGW7	7	SSR7-76	56.1	2.96	—	—	—	0.04	—	—	—	3.8	—	—	—
	qGW8	8	SSR8-417	90.3	11.32	6.32	3.49	7.22	0.08	0.07	0.06	0.06	16.5	14.1	9.8	15.2
	qGW12	12	STS12-8	57.2	7.01	—	—	—	−0.06	—	—	—	9.6	—	—	—
LWR	*qLWR1-1*	1	SSR1-312	38.4	—	—	—	3.13	—	—	—	−0.03	—	—	—	5.8
	qLWR1-2	1	SSR1-104	167.9	3.74	11.53	—	5.33	0.05	0.08	—	0.05	7.9	18.9	—	10.2
	qLWR2-1	2	SSR2-71	16.8	—	4.51	—	—	—	0.09	—	—	—	6.6	—	—
	qLWR2-2	2	STS2-479	59.3	—	—	—	4.35	—	—	—	0.05	—	—	—	8.2
	qLWR3-1	3	SSR3-288	17.1	—	2.83	—	3.76	—	0.04	—	0.05	—	4.1	—	7.1
	qLWR3-2	3	SSR3-120	41.6	3.75	6.04	4.52	6.01	0.04	0.04	0.05	0.04	7.9	9.1	11.4	11.6
	qLWR3-3	3	SSR3-35	144.3	4.78	—	—	—	−0.08	—	—	—	10.3	—	—	—
	qLWR10-1	10	SSR10-79	20.1	3.36	5.26	—	—	−0.04	−0.04	—	—	7.1	7.8	—	—
	qLWR10-2	10	SSR10-169	80.7	—	—	2.53	—	—	—	0.05	—	—	—	6.2	—
	qLWR11	11	SSR11-324	97.9	—	3.45	—	—	—	0.03	—	—	—	5.0	—	—

(Continued)

Table 2 (continued)

Traits	QTL	Chr.	Marker name	Position (cM)	LOD				Additive effect				PVE (%)			
					2015SH	2015LS	2016SH	2016LS	2015SH	2015LS	2016SH	2016LS	2015SH	2015LS	2016SH	2016LS
GP	*qGP1*	1	STS1-12	132.6	—	2.63	—	—	—	0.32	—	—	—	1.9	—	—
	qGP2	2	SSR2-24	92.2	4.37	9.75	3.67	7.43	−0.22	−0.32	−0.20	−0.28	7.9	7.8	4.6	16.3
	qGP3-1	3	STS3-1	113.8	—	3.96	12.16	2.95	—	0.21	0.41	0.18	—	2.9	17.4	6.0
	qGP3-2	3	SSR3-337	132.3	—	—	5.78	—	—	—	−0.31	—	—	—	7.5	—
	qGP3-3	3	SSR3-36	147.7	5.22	—	—	—	−0.49	—	—	—	9.5	—	—	—
	qGP8-1	8	STS8-3	49.0	—	21.43	—	—	—	0.57	—	—	—	20.7	—	—
	qGP8-2	8	SSR8-538	70.6	—	12.39	—	—	—	−0.45	—	—	—	10.3	—	—
	qGP8-3	8	SSR8-417	90.3	5.82	—	—	—	0.26	—	—	—	10.6	—	—	—
	qGP8-4	8	SSR8-390	112.0	—	—	3.95	—	—	—	0.25	—	—	—	5.0	—
	qGP11-1	11	STS11-2	23.6	—	—	3.71	—	—	—	0.23	—	—	—	4.7	—
	qGP11-2	11	SSR11-113	60.0	3.88	—	—	—	0.23	—	—	—	6.9	—	—	—
	qGP12	12	SSR12-101	66.4	3.15	4.61	—	4.43	−0.23	−0.27	—	−0.27	5.5	3.4	—	9.3
GA	*qGA2*	2	SSR2-24	92.2	7.99	10.65	7.32	13.13	−0.49	−0.54	−0.49	−0.60	10.5	7.2	9.0	22.3
	qGA3-1	3	STS3-1	113.8	9.75	4.03	14.07	3.01	0.59	0.35	0.77	0.29	13.2	2.5	19.2	4.4
	qGA3-2	3	SSR3-337	132.3	3.83	—	3.92	—	−0.40	—	−0.43	—	4.7	—	4.6	—
	qGA8-1	8	STS8-3	49.0	—	29.34	4.03	5.80	—	1.17	0.39	0.42	—	27.1	4.7	8.8
	qGA8-2	8	SSR8-538	70.6	—	13.97	—	—	—	−0.78	—	—	—	10.0	—	—
	qGA8-3	8	SSR8-417	90.3	7.39	—	—	—	0.48	—	—	—	9.7	—	—	—
	qGA8-4	8	SSR8-390	112.0	4.25	—	3.26	—	0.43	—	0.39	—	5.5	—	3.8	—
	qGA11-1	11	SSR11-235	3.6	—	2.62	—	—	—	0.26	—	—	—	1.6	—	—
	qGA11-2	11	STS11-2	23.6	4.42	—	4.26	—	0.41	—	0.42	—	5.5	—	5.0	—
	qGA12	12	SSR12-101	66.4	5.45	3.96	—	5.28	−0.51	−0.40	—	−0.46	6.9	2.4	—	7.9
TGW	*qTGW2-1*	2	SSR2-24	92.2	3.37	7.30	3.47	9.81	−0.79	−1.03	−0.68	−1.16	6.8	13.4	6.8	17.5
	qTGW2-2	2	SSR2-213	110.0	—	—	6.18	—	—	—	0.98	—	—	—	12.6	—
	qTGW3	3	SSR3-36	147.7	2.56	—	—	—	−1.42	—	—	—	5.1	—	—	—
	qTGW6	6	SSR6-111	6.3	3.42	2.98	6.20	2.74	0.70	0.56	0.82	0.51	6.9	5.1	12.6	4.4
	qTGW8-1	8	STS8-3	49.0	—	5.08	—	4.97	—	0.94	—	0.88	—	9.0	—	8.2
	qTGW8-2	8	SSR8-170	104.9	3.95	—	—	—	1.15	—	—	—	8.0	—	—	—
	qTGW10	10	STS10-4	29.3	—	—	3.44	—	—	—	0.75	—	—	—	6.7	—
	qTGW11	11	STS11-2	23.6	3.98	—	—	—	0.99	—	—	—	8.1	—	—	—
	qTGW12	12	SSR12-101	66.4	—	—	—	2.62	—	—	—	−0.72	—	—	—	4.2

Note: GL: Grain length; GW: Grain width; LWR: Length width ratio; GP: Grain perimeter; GA: Grain area; TGW: Thousand grain weight; Chr.: Chromosome; PVE: Phenotypic variance expressed.

For GP, a total of twelve QTLs located on chromosomes 1, 2, 3, 8, 11, and 12 were detected (Table 2). *qGP2* was identified on chromosome 2 under four environments, and the highest phenotypic variation of *qGP2* was detected in Lingshui 2016, reaching 16.3%. The alleles decreased GP were contributed by Koshihikari. *qGP3-1* and *qGP12* were both detected under three environments, and explained 2.9%, 17.4%, 6.0% and 5.5%, 3.4%, 9.3% of the phenotypic variance in Lingshui 2015, Shanghai 2016, Lingshui 2016 and Shanghai 2015, Lingshui 2015, Lingshui 2016, respectively. Another QTL, *qGP8-1*, was only detected in Lingshui 2015, which showed the highest phenotypic variation for GP, reaching to 20.7%.

Ten QTLs located on chromosomes 2, 3, 8, 11, and 12 were detected for GA. Those QTL carried the LOD score values from 2.62 to 29.34, with explained phenotypic variation in the range of 1.6% to 27.1% (Table 2). *qGA2* and *qGA3-1* were detected under four environments, with individually accounted for 10.5%, 7.2%, 9.0%, 22.3% and 13.2%, 2.5%, 19.2%, 4.4% of the phenotypic variation, respectively. *qGA8-1* and *qGA12* were both detected under three environments, and the highest phenotypic variation of those two QTLs was detected in Lingshui 2015 and Lingshui 2016, which reached 27.1% and 7.9%, respectively. In the remaining six QTLs, three QTLs were detected under two environments, and others were detected in one environment.

A total of nine QTLs were detected on chromosomes 2, 3, 6, 8, 10, 11, and 12 for TGW (Table 2). The QTLs, *qTGW2-1 and qTGW6*, were detected under four environments, with individually accounted for 6.8%, 13.4%, 6.8%, 17.5% and 6.9%, 5.1%, 12.6%, 4.4% of the phenotypic variation, respectively. The highest phenotypic variation of those two QTLs was detected in Lingshui 2016 and Shanghai 2016, which reaching to 17.5% and 12.6%, respectively. The allele of *qTGW2-1* from Koshihikari decreased TGW by about 1.03% in Lingshui 2015 and 1.16% in Lingshui 2016, and the allele of *qTGW6* from Nona Bokra increased TGW about 0.56% in Lingshui 2015 and 0.51% in Lingshui 2016. Other seven QTLs for TGW, one was detected under two environments, and the other six were detected in one environment. The LOD score values of those QTLs ranged from 2.56 to 6.18, with individually accounted for 4.2% to 12.6% of the phenotypic variation.

3.4 Digenic Epistasis QTLs for Grain Size Traits

To further understand the genetic components of grain size traits, the digenic epistatic QTL of GL, GW, LWR, GP, GA, and TGW was estimated. A total of five pairs of digenic epistatic QTLs were detected except for GL and GP (Table 3). There is no significant digenic epistatic QTL was detected for GL and GP, which indicated that the main effect of QTL is the primary genetic basis for GL and GP. One pair of digenic epistasis QTL was detected for GW, LWR, and GA, which explained 19.86%, 20.79%, and 21.25% of the phenotypic variation, respectively. Two pairs of digenic epistatic loci for TGW were estimated and accounted for 19.18% and 14.92% of the phenotypic variation, respectively.

Table 3: Epistasis effect for grain size traits in CSSLs population of Koshihikari and Nona Bokra

Trait	Chr.	Marker	Chr.	Marker	LOD	Epistasis (AA)	PVE (%)
GW	7	SSR7-4	8	SSR8-417	10.71	0.07	19.86
LWR	1	SSR1-104	8	SSR8-235	10.86	0.04	20.79
GA	4	STS4-11	8	SSR8-417	10.14	0.42	21.25
TGW	2	SSR2-24	8	STS8-1	10.88	0.92	19.18
TGW	2	SSR2-24	10	SSR10-169	12.97	0.80	14.92

Note: GL: Grain length; GW: Grain width; LWR: Length width ratio; GP: Grain perimeter; GA: Grain area; TGW: Thousand grain weight; Chr.: Chromosome; PVE: Phenotypic variance expressed.

3.5 Potential Candidate Gene for QTL Mapping

According to the results of QTL mapping, we further integrated the known grain size genes and QTL mapping in this study. One known grain size gene, *qGL3*/*GL3.1*/*OsPPKL1* is located near to the marker STS3-1 on chromosome 3 (Fig. 3). *qGL3*/*GL3.1*/*OsPPKL1* encodes a type 2A phosphatase and function as a negative regulator for regulating rice grain length [25–27]. We further compare the sequence of *qGL3*/*GL3.1*/*OsPPKL1* between the two parents, a T/C non-synonymous change in the 11th exon was identified (Fig. S1). According to previous studies, this C/T non-synonymous change can influence the function of *GL3.1*, thereby affecting grain length [26].

4 Discussion

4.1 Compared with Previously Identified QTLs or Genes

In the present study, we identified sixty-four QTLs for six grain size traits, including fourteen QTLs for GL, nine QTLs for GW, ten QTLs for LWR, twelve QTLs for GP, ten QTLs for GA, and nine QTLs for TGW (Table 2). Of these, thirty-eight QTLs caused an increase and twenty-six QTLs caused a decrease in the corresponding grain traits in the CSSLs. This suggests that grain size traits are controlled by complex genetic mechanisms. A number of QTLs affecting grain size traits have been mapped previously. For GL, eleven of the fourteen QTLs (*qGL2-1*, *qGL2-2*, *qGL3-2*, *qGL3-3*, *qGL3-4*, *qGL3-5*, *qGL4*, *qGL8-1*, *qGL8-2*, *qGL10*, and *qGL12*) were located in the same regions or close to previously reported [12,64–68]. The known grain size gene, *qGL3*/*GL3.1*/*OsPPKL1*, was located in the region of *qGL3-3* [25–27]. The sequence analysis of *qGL3*/*GL3.1*/*OsPPKL1* revealed that a single base-pair substitution between Koshihikari and Nona Bokra was found (Fig. S1). For GW, seven of the nine QTLs (*qGW1-1*, *qGW1-2*, *qGW2-1*, *qGW2-2*, *qGW6*, *qGW7*, and *qGW8*) were located in the same regions or close to previously reported [49,64,65,67–69]. But no known grain size gene was located in those QTLs regions. The QTL, *qGW8*, located on chromosome 8 was detected under four environments and the phenotypic variation was range from 9.8%–16.5%, suggesting a potential gene controlling grain width may exist in this region. Four of the ten QTLs for LWR (*qLWR1-1*, *qLWR2-2*, *qLWR3-1*, and *qLWR3-3*) were located in the same regions with previously reported [65,67,69]. The remaining six QTLs have not been reported before, especially for *qLWR1-2* and *qLWR3-2*, which were detected at least under three environments, suggesting a potential gene controlling length-width ratio may exist in those two regions (Table 2). Similarly, for TGW, six of the nine QTLs (*qTGW2-2*, *qTGW3*, *qTGW8-1*, *qTGW8-2*, *qTGW10*, and *qTGW11*) were located in the same regions to previously reported [49,64,70]. Of the remaining three QTLs, *qTGW2-1* and *qTGW6* were detected under four environments, but previous studies have not detected these two QTLs, suggesting a potential gene controlling thousand grain weight may exist in those two regions (Table 2). A total of forty-two QTLs were detected in the above four traits, of which fourteen QTLs were not previously reported, indicating that QTL mapping using CSSLs population can reduce the interference of background noise and make some minor QTLs could be detected. In addition, we found that some known grain size genes, such as *GS3*, *GW5*, *GL7*, etc., were not detected in this population, one possible reason is that the two parents harbor the same allele.

GP and GA are also important agronomic traits associated with grain size, but no studies on QTL mapping of rice GP and GA have been conducted. In our study, a total of twelve and ten QTLs were detected for GP and GA, respectably (Table 2). Especially for *qGP2*, *qGA2*, and *qGA3-1*, these three QTLs were detected under four environments, which suggests that these QTLs were stable and worth further fine mapping and cloning in the future.

4.2 Multi-trait QTLs and Digenic Epistatic QTLs Contributing to Grain Size Traits

It had been common in rice to find QTLs for different traits located in the same regions [68]. Multi-trait QTLs is contributing to the complex correlations of grain size traits. In this study, thirteen multi-trait QTLs

were found on chromosomes 1, 2, 3, 8, 10, 11, and 12 (Table 2, Fig. 3). We found that 16, 4, 3, 3, and 8 QTLs mapped on marker SSR2-4, SSR3-337, SSR3-36, SSR10-79, and SSR12-101, respectively, and their additive effect all caused a decrease in the corresponding grain traits. Similarly, 5, 3, 5, 9, 7, 6, 4, and 4 QTLs mapped on marker SSR1-104, SSR3-288, SSR3-120, STS3-1, STS8-3, SSR8-417, SSR8-390, and STS11-2, respectively, but their additive effect all caused an increase in the corresponding grain traits.

The digenic epistatic QTL for six grain size traits also have been estimated, and digenic epistatic QTLs for four traits were identified except for GL and GP (Table 3). Although only five digenic epistatic QTLs were detected, the phenotypic variation was in the range of 14.92% to 21.25%, which indicated that epistatic QTLs were also played an important role for grain size traits. Especially for the QTLs located on three markers SSR1-104, SSR2-24, and SSR8-417, they are both epistatic QTL and the multi-trait QTL, which reminds us to consider the epistatic effects and multiple effects during gene pyramiding in rice breeding.

4.3 Potential Utilization of CSSLs and QTLs in Rice Breeding

In our study, the CSSLs population showed a wide phenotypic variation and exhibited transgressive segregants in all tested environments (Table 1, Fig. 1). Therefore, it may be the best breeding material for breeders to improve grain size traits. The thirteen multi-trait QTLs identified in this study can be targeted for the improvement of rice grain yield and appearance quality. In addition, seven QTLs stably expressed in four conditions detected in this study lay the foundation for further validation, fine mapping and positional cloning.

5 Conclusion

In this study, the grain size traits including GL, GW, LWR, GP, GA, and TGW were investigated using a CSSLs population. We identified 64 QTLs for 6 grain size traits, of which 36 QTLs were novel and 7 QTLs were identified under four environments. The known grain size gene, *qGL3*/*GL3.1*/*OsPPKL1* is located near the marker STS3-1 on chromosome 3, showed a non-synonymous change between two parents. Moreover, five pairs of digenic epistatic QTL were identified except GL and GP. A highly phenotypic variation of digenic epistatic QTL indicated that the epistatic effects should be considered during rice breeding.

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Appendix

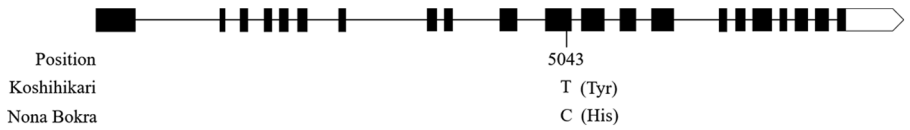

Figure S1: Sequence alignment in *qGL3*/*GL3.1*/*OsPPKL1* between Koshihikari and Nona Bokra. The number and single letter indicate the base position and difference, respectively. The letters in parentheses indicate amino acids

Table S1: The primer sequences used in this study

Primer name	Forward primer	Reverse primer
GL3.1-1	AAACCACCCGACGAATCCGC	AATATCCACGAGACACGACCAA
GL3.1-2	AGGCACGACTCTGATATGAAATT	TGGCGGTCCTTCACCTTCTGGT
GL3.1-3	CATCTTCTGAGGTGCTAATGTTT	GACAGCCAAGTGTTATACATTCA
GL3.1-4	GTCTAGTTATCCTTATATTCGGCA	TTACCTCCTCGTAGACCTCCATA
GL3.1-5	CTATCTGGTTCAGTGCTAGACA	TGAGGCTTGAATGCTTGCTGGTC
GL3.1-6	TGTGCTTCAGGGACAGAGGTTAT	TTAAAAAGTGTGTGATTACTAG
GL3.1-7	ACTGCTTTGGCACTAGGATTTAG	ATTCGCTCTATGCACTCTATTCG
GL3.1-8	CCTTTACGAGTATGGAGGGAGT	CTCCATAACACGATCAGGCTGTA
GL3.1-9	TATAGGTCTGATCCAACCGAGAA	CTATCCACCAGCAGCCGAGTA