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Validation of QTLs associated with yield and yield related traits in rice (*Oryza sativa* L.)

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Abstract
A previous study conducted on a new plant type based recombinant inbred line mapping population derived from Pusa 1266 X Jaya identified nine major quantitative trait loci (QTLs) expressing across environments for nine yield and yield component traits. For utilization of the markers flanking the major QTLs for marker assisted improvement, we analyzed SSR markers flanking QTL regions in a set of 120 germplasm lines to find marker QTL association. Out of eighteen markers linked to nine QTLs representing nine traits, seven markers linked to six QTLs showed significant association with respective traits in germplasm lines. Two flanking markers, RM152 and RM25 linked to a QTL *qDFF8-1* was significantly associated with days to 50% flowering at P≤0.01 level and explained 6% and 16% of phenotypic variation in the germplasm lines respectively. A QTL affecting panicles per plant (*qPPP4-1*) flanked by marker RM262 showed significant association in the germplasm lines. The genomic region showing pleiotropic effect on flag leaf width (*qFLW4-2*), spikelets per panicle (*qSPP4-1*), filled grains per panicle (*qFGP4-2*) and spikelet setting density (*qSSD4-2*) flanked by SSR marker RM3276 was significantly associated at P≤0.01 explaining 9%, 6%, 9% and 12% of phenotypic variation respectively in germplasm lines. The markers RM25, RM262 and RM3276 showing significant association of genomic region in germplasm lines can be utilized for marker assisted improvement of the component traits of yield in rice.

Keywords: validation, SSR markers, QTLs, yield, rice

Introduction
In recent years with availability of DNA markers and powerful biometrical methods, it has become feasible to identify the genetic factors underlying quantitative traits, commonly known as quantitative trait loci (QTLs). Meanwhile, QTL mapping has resulted in a large number of research papers with understanding about how many loci affect the traits, where are they located in the genome, and how do they interact. The information generated in QTL mapping experiment regarding markers linked to genomic regions effecting quantitative traits are useful for marker assisted selection and map based cloning. Major criteria for using the identified QTLs for marker assisted selection is that they should have significant effect, mapped with high degree of accuracy, should express across locations, effective in desired genetic background and should not have negative effect on other traits. It is necessary to validate these putative quantitative trait loci across various genetic backgrounds before using for marker assisted selection.

There are a few important factors that determine whether a QTL-maker association identified in one population will be useful for selection in a different population. These include the presence of marker-QTL segregation, the linkage phase of the marker and QTL and various other loci in the new population. Polymorphism of the markers is easy to determine, but the association of a given marker with quantitative trait in new population or germplasm may need to be determined experimentally [1]. One approach to validate marker QTL association across populations is to develop multiple mapping populations and to perform interval mapping on each to identify QTL, which are common to two or more populations [2]. Repeating the interval mapping experiment in an entirely new population to validate QTLs may be time consuming and expensive. Selective genotyping has shown to be nearly as effective as interval mapping for identifying QTLs [3]. Another approach for validation of QTLs is development of near isogenic lines by introgressing QTL region into new back ground by back cross breeding and using to precisely estimate effect of QTL region.

A previous study conducted on a new plant type based recombinant inbred line mapping

population derived from Pusa1266 X Jaya identified nine major quantitative trait loci (QTLs) expressing across environments for nine yield and yield component traits [4,5]. In the present study we analyzed SSR markers flanking major QTL regions influencing nine yield and yield component traits in a set of 120 germplasm lines to find marker-QTL association for utilization in marker assisted improvement of rice.

Materials and Methods

Plant material and phenotypic data

The plant material consisted of the a set of 120 germplasm lines, representing diverse genetic material including land races, improved varieties from North and South India, *japonica* lines covering short, medium, long duration groups, irrigated and rainfed ecotypes, basmati, non basmati grain characteristics, and resistant to different biotic and abiotic stresses (Table 1). One hundred and twenty germplasm lines were transplanted in randomized complete block design. Each entry was transplanted with three rows each in two replications. Each row consists of 15 plants with spacing of 30 cm between rows and 20 cm between plants. Five single plants were selected from the middle of the row avoiding border plants for data collection. Data on days to 50% flowering (DFF), panicle length (PL), panicles per plant (PPP), flag leaf width (FLW), spikelets per panicle (SPP), filled grains per panicle (FGP), percent sterility, 1000 grain weight and spikelet setting density (SSD) was collected as per Standard evaluation system of rice.

Table 1: List of germplasm lines used for validation of yield QTLs in rice

Sl. No	Variety	Sl. No	Variety	Sl. No	Variety	Sl. No	Variety
1	Sathi	31	Adt 38	61	Taipai-309	91	Pothana
2	Heera	32	Mandya Vijya	62	Pechi Badam	92	JGL3844
3	IR-20	33	Kanak	63	Golmalati	93	JGL13595
4	Narendra-118	34	PR 106	64	Hasan Sarai	94	RNRM7
5	IR 36	35	Pusa 1342	65	Tetep	95	Rajavadlu
6	IR 50	36	PR 113	66	Sonasal	96	Sumati
7	Neela	37	Phalguna	67	P-1301(K02)	97	Saleem
8	ADT -37	38	Kanti	68	Second Basmati	98	NLR34449
9	VL Dhan -221	39	Pusa Basmati-1	69	Pusa Sugandh-3	99	NLR30491
10	Narendra-97	40	Lunishri	70	Kasturi	100	Swarnamukhi
11	Rasi	41	Pusa 1121	71	Basmati-370	101	Pelelavadlu
12	Red Triveni	42	JD-6	72	CSR -13	102	Divya
13	PNR 162	43	Hei-Bao	73	Tarori Basmati	103	Surekha
14	Jyoti	44	N-22	74	Pusa sungandh-2	104	Bhadrakali
15	PR 108	45	Pusa44	75	indli	105	Kavya
16	Annada	46	ADT43	76	ShaPasand	106	WGL14
17	Vikas	47	Kalinga-3	77	Intan	107	JGL3828
18	Ratna	48	Aditya	78	Pokali	108	JGL11727
19	Krishna Hamsa	49	Vanparva	79	Nipponbare	109	Sagarsamba
20	PNR -381	50	Tripura Medicinal Rice	80	P-1221	110	Chandana
21	Pusa 205	51	Jhum Khasa	81	CSR-36	111	Sonamahsuri
22	IR 4630	52	TKM-6	82	Tellahamsa	112	Sambamahsuri
23	JD10	53	Pant Dhan-12	83	Satya	113	Keshava
24	Pusa 169	54	Narendra 359	84	Rajendra	114	Orugallu
25	Pusa-834	55	SKR126	85	Rudrama	115	Shiva
26	IR-64	56	CSR-27	86	Indursamba	116	Pusa 1266
27	PR-111	57	MI-48	87	Varsha	117	Prabhat
28	Malviya Dhan-36	58	Pusa sungadh-5	88	Erramallelu	118	Vijetha
29	Vijram	59	Jaya	89	Varalu	119	Cottondora Sannalu
30	Pant Dhan-4	60	P-1447	90	WGL32100	120	MTU1081

DNA isolation, quantification and PCR

DNA was isolated by CTAB (Cetyl- Tetra Methyl Ammonium Bromide) method [6] and quantified using gel electrophoresis in 0.8% agarose gel in 0.5X TAE buffer along with known concentrations of λ genomic DNA as standards. The PCR reactions were carried out in 96-well PCR plates obtained from Axygen Scientific Inc., Union city CA, USA. The master mix consisted of 25 ng of genomic DNA, 0.2 U of *Taq* DNA polymerase, 1 X PCR assay buffer with 1.5 mM $MgCl_2$, 12 ng (1.8 picomole) each of forward and reverse primer and 200 µM of dNTP mix in a 10 µl reaction volume. The reaction mix was prepared on ice and the PCR plate was immediately loaded in the thermal cycler (Eppendorf, Biometra or Applied Biosystems USA) for PCR using conditions of (1) initial denaturation at 94°C for 5 min; (2) 35 cycles of 94°C for 1 min, 55-60°C (depending on marker) for 1 min, 72°C for 2 min; (3) final extension at 72°C for 5 min. The PCR products were separated on 3% MetaPhor® agarose gel and visualized in a UV transilluminator.

SSR markers and data analysis

A list of nine major QTLs identified in the previous study has been given in Table 2. A set of 18 markers linked to nine major QTLs were used to genotype germplasm lines. The Banding pattern of each marker was recorded for each genotype. SSR allele size was determined depending on the position of band relative to the ladder. The mean phenotypic data of 120 germplasm lines for nine yield component traits along with marker (linked to QTL) genotypic data was subjected to single marker analysis by using PROC GLM of SAS separately for each trait. The F probabilities and R^2 values generated were analyzed for declaring significant association of markers with phenotype in the germplasm set.

Table 2: QTLs expressing across three environments in Pusa1266 X Jaya population

Sl. No	Ch	QTL Name	Marker interval	Location			Marker position	Genetic interval	Physical interval	LOD		Additive		R^2	
				ND	KAR	ADT				MIN	MAX	MIN	MAX	MIN	MAX
1	8	*qDFF8-1*	RM152-RM25	*	*	*	82-190.3	108.3	4,540,944	3.05	22.8	1.73	7.26	0.04	0.55
2	2	*qPL2-3*	RM262-GNMS3876	*	*	*	85.4-107.8	22.4	9,454,047	3.36	7.74	-0.60	-0.74	0.07	0.10
3	4	*qPPP4-1*	RM241-GNMS1539	*	*	*	142.1-163.6	21.5	3,577,033	4.38	7.66	-0.7	-1.21	0.09	0.16
4	4	*qFLW4-2*	RM3276-RM5709	*	*	*	8-177.8	9.8	814,464	21.7	46.5	0.15	0.24	0.33	0.53
5	4	*qSPP1*	RM327RM5709	*	*	*	168-177.8	9.8	814,464	5.71	13.84	20.14	28.61	0.09	0.16
6	4	*qFGP4-2*	RM3276-RM5709	*	*	*	68-177.8	9.8	814,464	7.71	15.27	18.07	23.8	0.12	0.22
7	3	*qPSTE3-1*	RM7-RM1140	*	*	*	92.9-148.4	55.3	6,877,844	7.85	14.24	5.04	8.03	0.16	0.21
8	6	*qTGW5-1*	RM1776-RM413	*	*	*	35.4-66.3	30.9	1,403,676	4.07	12.88	-0.98	-1.37	0.12	0.25
9	4	*qSSD4-2*	RM3276-RM5709	*	*	*	68-177.8	9.8	1,314,464	7.99	16.6	0.92	1.20	0.13	0.19

Results and Discussion

The characteristics of 120 germplasm lines i.e., minimum, maximum, mean, range and standard error for nine yield component traits i.e., DFF, PL, PPP, FLW, SPP, FGP, PSTE, TGW and SSD is presented in Table 3.

Table 3: Characteristics of 120 germplasm lines used for validation of QTLs

Sl. No	Trait	Range		Mean	SEm±
		Min	Max		
1	Days to 50% flowering	46.00	124.00	90.82	0.85
2	Panicle length (cm)	19.00	35.70	25.72	1.28
3	Panicles per plant	5.50	19.50	12.12	2.39
4	Fleaf width (cm)	0.83	2.45	1.54	0.22
5	Spikelets per panicle	88.50	336.00	156.28	47.54
6	Filled grains per panicle	82.00	276.50	120.28	24.67
7	Percent sterility	3.29	37.68	27.16	9.59
8	Thousand grain weight (g)	9.65	34.12	20.11	2.30
9	Spikelet setting density	2.93	12.03	6.51	1.07

The trait DFF among germplasm lines ranged from 46 days in jaldi dhan-6 to 125 days in MTU1001 with mean of 91 days. The mean PL of germplasm lines was 25.72cm, while jaldi dhan-6 showed shortest panicle length (19.00cm) and kasturi had maximum panicle length (35.70cm) (Fig 1). Pusa1121, a popular basmati variety has maximum number of panicles (19.5) per plant whereas, tripura medicinal rice showed only 5.5 panicles per plant. Hasansarai, a land race, showed narrow flag leaf (0.83cm) whereas, Pusa 1266, which is one of the parent of RIL population in which QTLs were identified, has widest leaf (2.45cm). The total number of spikelets per panicle were minimum in divya (88.5) and maximum in pechibadam (336). TKM 6 had lowest filled grains per panicle (82.5) whereas, WGL 32100 had highest filled grains per panicle (276.5). The mean per cent sterility in germplasm lines was 27.16 % with a minimum of 3.29% in WGL 32100 and maximum of 37.68% in TKM 6. The 1000-grain weight of sagarsamba variety was minimum (9.65g) whereas, shahpasand had a maximum test weight (34.12g). Among germplasm lines, WGL32100 recorded maximum spikelet setting density (12.03 grains per cm length of panicle) and it was minimum in P1301 (2.39).

Fig 1: Variation for panicle length and number of grains per panicle of germplasm lines

Genotyping of 120 germplasm lines was done with eighteen markers on five chromosomes. A gel picture showing amplification pattern in germplasm lines is given in Fig. 2.

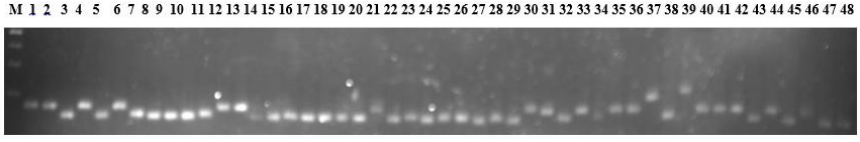

Fig 2: Gel picture showing amplification pattern of RM413 in germplasm lines

M- 100bp ladder; 1-48 – Germplasm line numbers

Genotypic data of eighteen markers linked to 9 QTLs along with phenotypic data of 120 germplasm lines were used to find marker QTL association with single marker analysis by using SAS v9.1 software. In association analysis of markers linked to QTLs, out of eighteen markers linked to nine QTLs representing nine traits, seven markers linked to six QTLs showed significant association with respective traits in germplasm lines explaining 6-16% of phenotypic variation (Table 4). Markers linked to QTL *qDFF8-1* i.e., RM152 and RM25 were significantly associated with days to 50% flowering at 1% level of significance and explained 6% and 16% of phenotypic variation in the germplasm lines respectively. For panicle length, two markers i.e., RM262 and

RM3876 on chromosome 2 were found to be non-significant association in germplasm lines. Out of two markers linked to panicles per plant, a marker RM241 linked to the QTL *qPPP4-1* has shown significant association with the trait in the germplasm lines and explained 5% phenotypic variation whereas other marker GNMS1539 has shown non-significant association. In a near isogenic F_2 population developed by using two plants of a RI line showing significant difference in heading date, panicle size and plant height has been used to validate the traits and found significant association[7]. Among the two markers RM3276 and RM5709 linked to QTL *qFLW4-2*, RM3276 was significantly associated with flag leaf width at 1% significance level and explained 9% of phenotypic variation in germplasm lines where as other marker, RM5709 has not shown any association. Out of two markers linked to spikelets per panicle, only one marker RM3276 linked to QTL *qSPP4-1* was significantly associated in germplasm lines at 0.01 level explaining 6% phenotypic variation. A RIL having QTL (*qSPP7*) allele of Minghui 63 with 70% of genetic background same as of female parent Zhenshan97 was crossed with Zhanshan97 to produce near isogenic lines for QTL region which have 51.1 and 24.9 spikelets as additive, dominant effects respectively [8]. Like markers linked to flag leaf width and spikelets per panicle, the same marker (RM3276) linked to QTL *qFGP4-2* effecting filled grains per panicle, was significantly associated in germplasm lines at 0.01 level explaining 9% phenotypic variation where as other marker, RM5709 has not shown any association. In another approach, an introgression line was developed by introgressing chromosomal segments (*gpa7*) from *O. rufipogon* into an *indica* cultivar Guichao2 and found that alleles from *O. rufipogon* decreased grains per panicle and this region influence five panicle traits showing pleiotropic effects [9]. For percent sterility, two markers i.e., RM7 and GNMs1140 on chromosome 3 have shown non-significant association in germplasm lines. In the same manner, two markers i.e., GNMS1776 and RM413 on chromosome 5 linked to *qTGW5-1* effecting thousand grains per panicle have shown non-significant association in germplasm lines. Similarly, association mapping was done for five traits i.e., grain yield, kernel length, width, length/width ratio, 1000 dehulled grain weight in a set of 103 germplasm lines with 123 marker, with fair amount of success establishing significant association between 25 markers with five traits and found that many of the associated markers were located in regions where QTL had previously been identified [10]. In rice, two sister BC_3F_3 Near isogenic lines developed from *Japonica* cultivar Hwaseongbyeo and *O. rufipogon* were used to estimate the effect of QTL affecting grain weight on chromosome 8 near SSR marker RM210 [11]. In another study carried out in rice, a natural grain weight mutant 'sgw' was crossed with a cultivar '9311' and in the population so derived, a QTL governing grain weight was detected on the short arm of chromosome 7. To validate and further refine the locus, QTL analysis based on F_2 and F_3 populations was conducted, and a single major QTL (designated as *qsgw7*) affecting the 1000-grain weight of paddy rice was identified on the short arm region of rice chromosome 7 between simple sequence repeat (SSR) markers RM21997 and RM22015.

Table 4: Validation marker- QTL association in germplasm lines by Single marker analysis

Sl. No	Trait	QTL	Markers	Distance from QTL (cM)	% variance	F Probability
1	Days to 50% flowering	*qDFF8-1*	RM152	33	6	0.01**
			RM25	18	16	0.0001**
2	Panicle length	*qPL2-3*	RM262	14	3	0.17^{NS}
			GNMS3876	6	2	0.22^{NS}
3	Panicles per plant	*qPPP4-1*	RM241	11	5	0.04*
			GNMS1539	9	1	0.91^{NS}
4	Flag leaf width	*qFLW4-2*	RM3276	2	9	0.003**
			RM5709	7	0.8	0.6^{NS}
5	Spikelets per panicle	*qSPP4-1*	RM3276	4	6	0.01**
			RM5709	5	0.7	0.65^{NS}
6	Filled grains per panicle	*qFGP4-2*	RM3276	4	9	0.003**
			RM5709	5	1	0.38^{NS}
7	Percent sterility	*qPSTE3-1*	RM7	30	3	0.11^{NS}
			GNMS1140	4	0.6	0.69^{NS}
8	1000-gain weight	*qTGW5-1*	GNMS1776	11	4	0.08^{NS}
			RM413	22	0.2	0.84^{NS}
9	Spikelet setting density	*qSSD4-2*	RM3276	2	12	0.009**
			RM5709	7	2	0.19^{NS}

The region so identified was novel and could affect grain weight and grain shape [12]. In an investigation taken up in rice, a RIL population derived from cross D50 (*javanica)* X HB277 (*indica*) was used to map seven QTLs for the trait thousand grain weight. Of all the loci, the QTL *qTGW3.2* located on chromosome 3 was stably expressed over two years and contributed 9-10% phenotypic variance. This region was validated in F_2 and $F_{2:3}$ populations derived from selfing a residual heterozygous line from the RIL population where it explained a variance of 23% and 33% respectively [13]. For spikelet setting density, a marker (RM 3276) linked to QTL *qSSD4-2* at 2 cM was also found to be significantly associated with spikelet setting density in germplasm lines explaining 12% of total phenotypic variation. In a research study carried out in rice to identify and validate QTLs controlling harvest index, the cultivars 'Yuexiangzhan' and 'Shengbasimiao' were crossed to develop a RIL population. QTL mapping resulted in identification of five QTLs for harvest index, three QTLs for grain yield, and six QTLs for biomass in two-year experiments. A harvest index QTL on chromosome 8, *qHI-8*, was detected across two years and explained 42.8% and 44.5% of the phenotypic variation, respectively. The existence of *qHI-8* was confirmed by the evaluation of the near isogenic lines derived from a residual heterozygous line, and this QTL was delimitated to a 1070 kb interval by substitution mapping [14].

In a study conducted in rice, a pair of SSR markers RM518 and RM225 reported in a RIL population derived from cross

Swarna x WAB450 flanking the QTLs for water use efficiency and nitrogen use efficiency respectively were used for validation in two F_2 populations derived from crosses BPT5204 x WAB450 and BPT5204 x Mysore Mallige. The markers RM518 and RM225 (for their respective traits) explained a phenotypic variance of 48.26% (p-0.0135) and 38.74% (p-0.0306) in the former and latter F_2 populations respectively [15]. In a work done in Africa, an F_2 population derived from a cross between Hnanomai (temperate *japonica*) and WAB56-104 (tropical *japonica*) was used to identify QTLs governing cold tolerance at booting stage. Two QTLs were detected on chromosome 8 and 10 which explained 30% and 33% variance respectively for spikelet fertility under cold. Selected BC_1F_4 and BC_1F_5 genotypes having homozygous alleles for both QTLs exhibited higher spikelet fertility under cold stress [16].

Repeating the interval mapping experiment in an entirely new population to validate QTLs may be time consuming and expensive. In the present study seven markers linked to six QTLs influencing six traits i.e. days to 50% flowering, panicles per plant, flag leaf width, spikelets per panicle, filled grains per panicle and spikelet setting density were validated in a set of 120 germplasm lines. However, the linked markers explained lesser variation in germplasm lines than mapping population. This might be due to use of *indica* germplasm lines in which alleles contributed by one of the parent Pusa1266, an *indica*/*japonica* derivative might not be widely distributed. Nevertheless, the alleles are novel and can be used for improvement of yield and its components. In this study, a marker interval RM3276-RM5709 on chromosome 4 influencing 4 traits namely flag leaf width (31-52%), spikelets per panicle (9-16%), filled grains per panicle (12-22%) and spikelet setting density (13-19%) showed significant association in germplasm. Out of seven flanking markers showing significant association, five markers are linked less than 10 cM away from QTL position may directly be used for marker assisted pyramiding of major QTLs for enhancing productivity of rice. The physical distance between flanking markers is about 1 Mb and may be fine mapped to identify candidate genes for all the four traits. There are number of reports of mapping and introgression of QTLs from wild species in rice. However, the related subspecies of *Oryza sativa* such as *japonica* carry many favorable alleles which can be used for improvement of *indica*. The markers RM25, RM262 and RM3276 showing significant association of genomic region in germplasm lines can be utilized for marker assisted improvement of the component traits of yield in rice.

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