Original Research Article

Parental Polymorphism Survey and Study of Allelic Variation for Grain Quality and Yield Traits using SSR Markers in Rice (*Oryza Sativa* L.)

ABSTRACT:

Improvement in rice grain quality traits particularly aroma and cooking and eating quality is an important breeding objective in rice. Identification of polymorphic markers is prerequisite for conducting any QTL mapping experiment because if the parents are polymorphic for the traits of interest, then further selection of plants in the progenies becomes easy. Hence, the objective of the present study was to identify polymorphic markers for grain quality and yield related traits among the parental lines Improved Samba Mahsuri and Badshabhog. For this a total of 576 randomly selected microsatellite markers distributed over all the 12 chromosomes of rice including 26 gene specific markers related to aroma, cooking and eating quality, grain dimension and yield related traits were used. Overall, 96 markers including 4 gene specific markers were found to be polymorphic between the two genotypes indicating a total polymorphism percentage of 16.67%. The highest polymorphism percentage was recorded on chromosome 6 (26.67%) followed by chromosome no 4 (21.43%) and the lowest polymorphism percentage was observed on chromosome 10 (8.93%). These markers will further be utilized in genotyping of $F_{2:3}$ population, linkage analysis and mapping QTL's for grain quality and yield traits.

Key words: *Rice, aroma, cooking and eating quality, parental polymorphism, QTL, SSR.*

1. INTRODUCTION:

Rice is an indispensable cereal to the world providing nutrition to about 21% of the global population and 2/3rd of the South East Asian population [24]. In 2019, global rice production was 755.47

million tons from 162.05 million ha of area. Among the 117 different rice producing countries, China ranks first with a production of 209.61 MT followed by India with 177.64 MT in 2019. Both these countries accounts for more than 50% of the total rice production. Asian countries contribute 89.6 % of the total world production (677.27 MT) followed by Africa (38.77 MT), America (35.32 MT), Europe (4.02 MT) and Oceania (0.76 MT) [2]. To meet the ever-increasing demand, several new varieties are breed every year to achieve the highest yield and are resistant to various biotic and abiotic stress also. But, the grain qualities in these varieties were less prioritized. Grain quality is very hard to define as it comprises of several different facets such as grain appearance quality, milling quality, nutritional quality, eating quality, and cooking quality [11,4]. The preferences and taste vary from one region to another, what may be preferable in one region may not necessarily be preferable in another region. [6]. Nearly 40% of people around the globe particularly in USA, Canada, Europe, Middle East and South East Asian countries desire to eat aromatic rice irrespective of grain types.

Most of the quality traits follow quantitative inheritance and have complex genetic architecture because of the considerable influence of genotype, environment, and their interactions [1,18]. Phenotype-based classical breeding approaches are time-consuming, laborious and are inefficient in improving traits that are governed by quantitative trait loci (QTL), such as yield and other grain quality traits which show continuous phenotypic variation and lack discrete phenotypic segregation in the progeny [32,22]. However, several recent developments such as the use of potential donor parents, identification of QTLs regions associated with important grain qualities, and identification of robust marker have increased our understanding of the genes, pathways, and molecular mechanisms determining overall quality traits in rice [11,20]. Breeding programs should be directed in to develop rice varieties with better eating quality and to meet out the demands of the local consumers as well as emerging food processing industry.

Mapping of quantitative trait loci (QTLs) for grain quality traits in rice is an important forward genetic approach and using these dissected complex regions in marker-assisted selection and gene discovery [27]. With the advent of several molecular techniques, the use of molecular markers has increased to a large extent in the recent past. Among the PCR-based markers, the SSR markers have proved to be very effective tools and first choice of breeders in the study of genetic diversity and organism relationships due to their several advantages such as they are co-dominant, highly polymorphic in nature,

evenly distributed in the genome, efficient, less quantity of DNA is required, are highly cost-effective and transferability [15]. These micro satellite markers can be effectively used in studying the genetic polymorphism i.e., occurrence of multiple alleles at a single locus, where at least two alleles occur with a frequency greater than 1% [12]. Also, they can be used in population structure analysis, gene mapping and tagging, linkage map construction, tracing marker-trait association, MAS, etc. [23]. So, developing rice varieties with desired grain quality i.e*.,* nutritional and cooking and eating quality which are high yielding as well as resistant to various abiotic and biotic stresses with the help of molecular techniques and breeding tools will help alleviate several malnutritional problem and will also fetch high price to farmers as well as food industries personnel. Thus, the present study was aimed to identify informative polymorphic SSR markers between two diverse parents viz Improved Samba Mahsuri and local rice germplasm Badshabhog for yield and quality traits in rice. The research was also planned to study the influence of the type of the repeat motif on polymorphism.

2. MATERIALS AND METHOD:

2.1 Plant Material used in the study:

The experimental plant material for this study comprised of a highly contrasting rice lines for desirable grain quality and yield traits. The first parent is short grain aromatic landrace 'Badshabhog', a popular and preferred line among the aromatic rice breeders and second variety is 'Improved Samba Mahsuri' a fine grain, medium slender non-aromatic rice, popular specially for grain quality. Details of all the traits of both the parents is presented in table 1. These two are popular varieties and being used as a source for desirable grain traits in rice breeding. Both the parents were raised by following all recommended package of practices as per recommendation of this zone. Twenty-one days old seedlings of Improved Samba Mahsuri and donor Badshabhog were transplanted in the main field for evaluation. Leaf samples were collected 14 days after transplanting and were preserved at -20^{o}C for parental polymorphism survey after DNA extraction.

Table 1. Details of the parents used in parental polymorphism experiment:

Sr.no	Trait name	Recipient	Donor
1	Name	Improved Samba Mahsuri	Badshabhog
2	Type	Released variety in 2008 jointly by ICAR-IIRR and CSIR-CCMB	Local landrace collected by Institute of Agricultural Sciences, BHU,

		Hyderabad, India	Varanasi, U.P, India
3	Parentage	Samba Mahsuri*4/SS1113	Local landrace
4	Yield	4.75-5.0 tonnes ha^{-1}	2.5-3 tonnes ha^{-1}
5	Days to 50 % flowering	100-103 days	120-123 days
6	Days to maturity	130-134 days	150-154 days
7	Plant height	100-103 cm	155-160 cm
8	Grain length	8.15 mm	5.53 mm
9	Grain breadth	2.01 mm	2.11 mm
10	Kernel length	5.32 mm	3.83 mm
11	Kernel breadth	1.78 mm	1.85 mm
12	Aroma	Non aromatic	Highly aromatic
13	Amylose content	23.84	18.46

2.2 Genomic DNA Isolation and quality check:

The genomic DNA of both the parents were extracted by the CTAB method [16], with some modifications. 100 mg of young leaves was weighed and the genomic DNA was extracted with DNA extraction buffer (10% CTAB, 1 M Tris HCl (pH 8.0), 0.5M EDTA (pH 8.0), 5 M NaCl, and 0.2 % β-mercaptoethanol) preheated at 60^{o} C. The quality and quantity of extracted DNA was judged on 0.8 % agarose gel electrophoresis along with a standard ladder and compared with band intensity and thickness. DNA quantification and purity were checked by measuring the O.D values at 260 and 280 nm using a NanoDrop ND100 spectrophotometer. The ratio of UV absorbance at A_{260}/A_{280} ranged between 1.89-1.96, and this ratio indicates good-quality DNA. The quantity of DNA in the isolated samples ranged from 1288.50 to 1701.30 ng/µl. After quantification, the DNA samples were diluted with TE buffer, so as to make final concentration of the DNA as 50 ng / µl stored at 4^{o} C.

2.3 Details of SSR markers used in the study:

A total of 576 randomly selected microsatellite markers including 26 gene specific markers covering the entire length of each of 12 chromosomes of rice were used to identify polymorphism between Improved Samba Mahsuri and Badshabhog. The information regarding chromosomal location, sequences of primers (forward and reverse), physical position (SSR start and SSR end), number of repeat motifs were obtained from Gramene markers database (https://www.gramene.org/). RAP-DB (The Rice Annotation Project Data Base) was also used for those markers whose information was not available in the Gramene database, by using its BLAST tool and submitting query sequence in FASTA format to run a search against DNA database (blastn) (https://rapdb.dna.affrc.go.jp/tools/blast).

2.4 PCR amplification:

PCR was carried out in in Eppendorf thermal cycler with a final reaction volume of 15 µl containing 1.2µl of genomic DNA, 1.5 µl of 10 X Taq assay buffer containing 15 mM $MgCl_2$, forward and reverse primer each 1 µl (10 pmol/ µl), 0.2µl of dNTPs (3 mM), 0.2µl Taq DNA polymerase (GeNei) and 9.9µl of HPLC water. The PCR reaction was performed under the following conditions: Initial denaturation at 94^o C for 5 min, followed by 35 cycles of denaturation at 94^o C for 45 seconds, annealing at the temperature specific to primer for 30 seconds, extension at 72^o C for 30 seconds, followed by the final extension at 72^o C for 5 min and hold at 4° C for ∞. After completion of amplification, PCR products were stored at -20° C

2.5 Agarose gel electrophoresis:

The final amplified products were analyzed by electrophoresis using 2.5% agarose gels in 1X TAE Buffer at 65V for initial 30 minutes followed by 90V for 1.5 hours. Gel was stained by Ethidium bromide (10 mg/ml) @ 2.5 µl/1000 ml 1X TAE Buffer. The DNA fragments were then visualized under Gel documentation system (Gel Doc TM XR+, BIO-RAD, USA) and the banding pattern was observed and recorded for further use.

3. RESULTS AND DISCUSSION:

In the present study, the parental polymorphism survey between the parents, Improved Samba Mahsuri and Badshabhog indicated a clear polymorphism and it is very important to understand this variation at molecular level. A total of 576 randomly selected SSR primers including 26 gene specific markers distributed all over 12 chromosomes were used for testing polymorphism between two parents. This was confirmed and tested by using graphical genotypes (GGTs) software package 2.0.[29]. Fig. 1 depicts the distribution of all the identified polymorphic markers across the length of 12 rice chromosome, according to their physical positions (Mb) on a genetic map. Of the 576 SSR markers used, 96 markers including 4 gene specific markers were found to be polymorphic between the two genotypes indicating a total polymorphism percentage of 16.67%. Some of the identified polymorphic markers are displayed in gel picture Fig. 2. Of all the chromosomes highest polymorphism percentage was recorded on chromosome 6 (26.67%) followed by chromosome no 4 (21.43%) and the lowest polymorphism percentage was

observed on chromosome 10 (8.93%). The chromosome wise polymorphism percentage is stated in table 2. For QTL mapping genetically diverse parents are required to map the traits of interest [9] and a high percentage of polymorphism between the two parents used in the study shows that the two parents are genetically diverse. The identified polymorphic markers can further be effectively used in QTL mapping experiment for preparation of linkage map and identification of QTL's related to grain quality and yield traits in mapping population derived from the above cross.

Table 2. Chromosomal wise polymorphism percentage of SSR markers between the parents Improved Samba Mahsuri and Badshabhog

Chromosome no.	Total No. of SSR marker used	No. of Polymorphic markers obtained	Polymorphism (%)
Chromosome 1	51	10	19.61
Chromosome 2	47	7	14.89
Chromosome 3	55	8	14.55
Chromosome 4	42	9	21.43
Chromosome 5	52	8	15.38
Chromosome 6	**30**	**8**	**26.67 (Highest)**
Chromosome 7	45	8	17.78
Chromosome 8	53	11	20.75
Chromosome 9	48	8	16.67
Chromosome 10	**56**	**5**	**8.93 (Lowest)**
Chromosome 11	45	7	15.56
Chromosome 12	52	7	13.46
Total	**576**	**96**	**16.67**

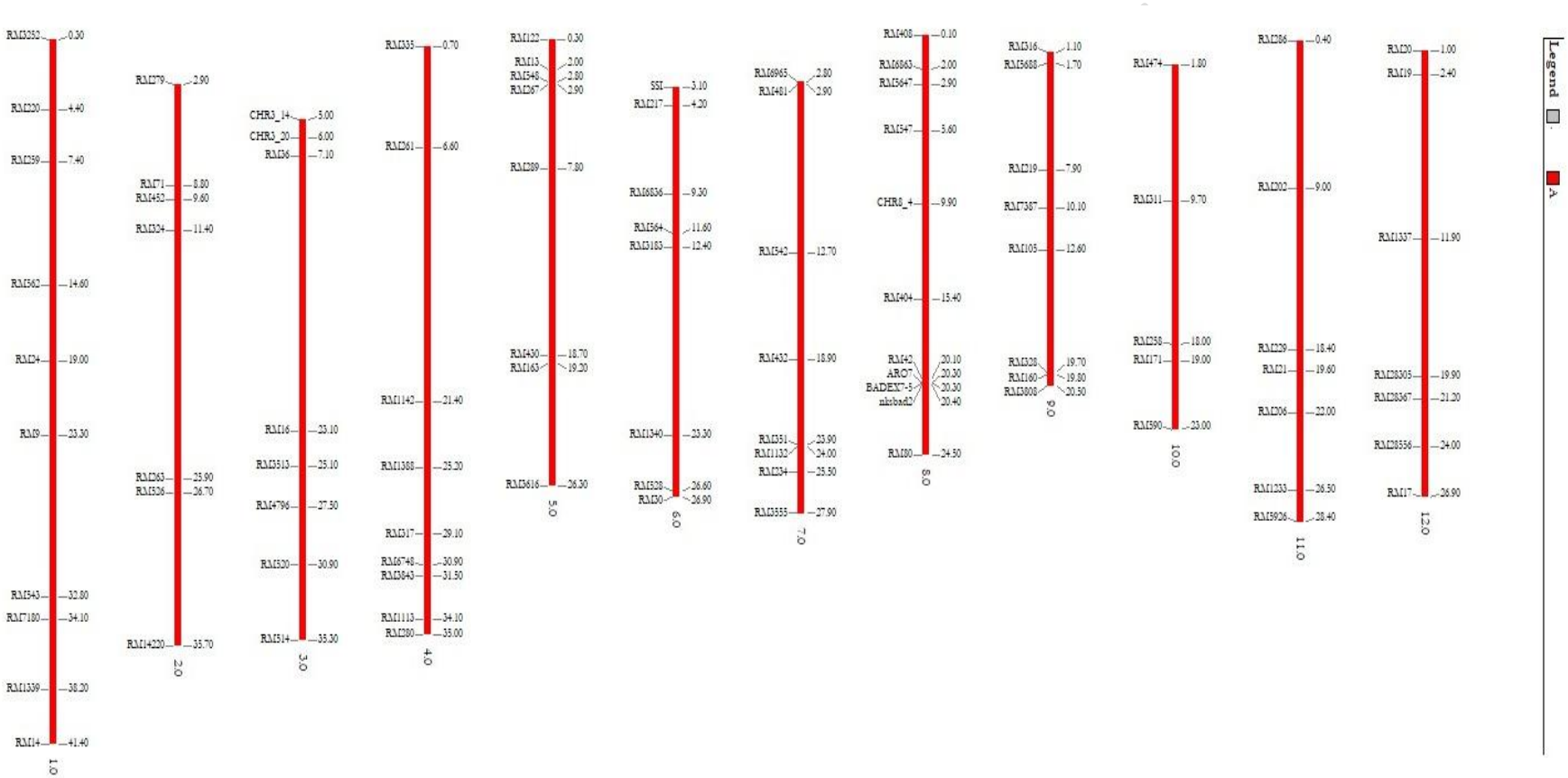

Fig. 1. Genetic linkage map showing distribution of polymorphic markers across the 12 chromosomes.

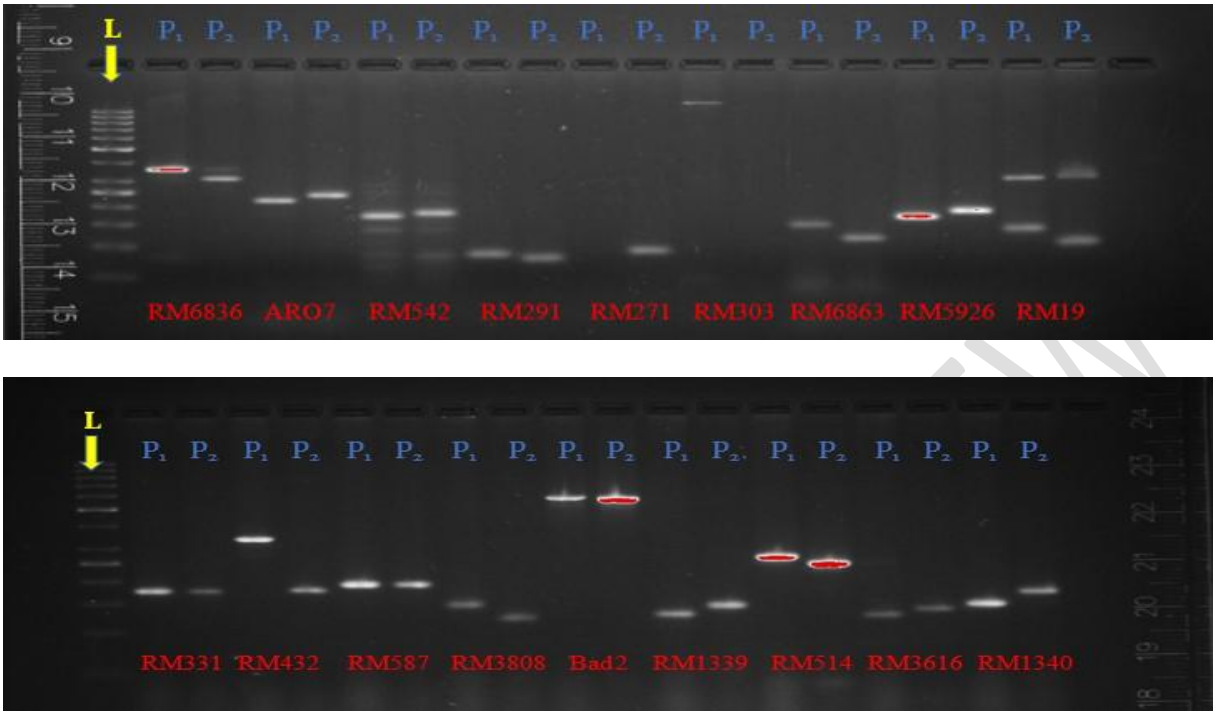

Fig. 2. Gel picture showing parental polymorphism survey with SSR markers among the parents. P1- Improved Samba Mahsuri, P2- Badshabhog, L- Ladder 50 bp.

Similar results were reported by other authors also. The polymorphism percentage highest for chromosome 6 and lowest on chromosome 10 was also reported by [23]. Similarly, [13] reported polymorphism percentage of 29.02% between the two parents Rajendrakasturi and URG-30. The highest polymorphism was reported on chromosome 4 (40.96%) whereas the lowest polymorphism was observed in chromosome 9 (16%). [7] found a total polymorphism percentage of 20.75% by using 800 SSR markers between BPT5204 and *O. rufipogon* WR119. A polymorphic percentage of 6.93 was reported by [14] using 1,904 genomic SSRs markers among the parents IR58025A and KMR-3R.

In the present study a total of 96 polymorphic markers were identified, out of which 20 SSR markers were reported to be associated with QTLs for different quality traits and the 4 gene specific markers were associated with genes of aroma and eating and cooking quality traits of rice from previous studies. Of the 4 gene specific markers 3 markers were found to tightly linked with the genes associated with aroma content in rice viz., ARO7 [26], BADEX7_5 [21], nksbad2 [1], and one marker SSI [3] was found to be associated with cooking and eating quality traits. The gene specific primer ARO7 is linked

with *fgr* gene encoding BAD2 which is associated in the expression of aroma in rice, while BADEX 7_5 and nksbad2 is linked to badh2 gene, with 8-bp deletion in the exon 7 of badh2 gene implicates with rice aroma. The gene specific primer SSI is linked to gene encoding granule-bound starch synthase I. The SSR markers RM19, RM217, RM219 flank the QTL associated with cooking and eating quality traits such as amylose content and alkali digestion value and RM19 was also linked with the trait kernel length after cooking [5]. [31] found that RM488, RM562, RM432 were linked with grain dimension traits such as grain length and grain breadth while RM432 was also found to be associated with amylose content in rice. [10] reported that RM7180, RM3616, RM6836, RM3183 were significantly associated with grain dimension traits such as grain length and grain breadth. Three SSR markers CHR3_14, CHR3_20, CHR8_4 was found to be associated with aroma content in rice [25]. Two SSR markers RM404, RM547 were found to be linked with aroma content and RM17 with amylose content in rice in a study conducted by [30]. [1] reported that RM80 and RM252 was found to be associated with aroma content in rice and RM217 and RM432 with cooking quality traits such as amylose content and alkali digestion value and grain dimension traits such as grain length and grain breadth. One SSR marker RM42 of chromosome8 was found to be linked with grain aroma content in two studies conducted by [8]. While [27] reported that RM5688 was found to be associated with amylose content and other cooking quality traits on chromosome 9.

Effect of the nature and form of the motif on polymorphism detection:

Within the set of 96 polymorphic marker, most consisted of dinucleotide motifs (52.08%), followed by trinucleotide motifs (22.92%), subsequently by tetranucleotide motifs (7.29%). Similarly group of either mono-dinucleotide (6.25%), mono-trinucleotide (1.04%), di-trinucleotide (2.08%), di-tetranucleotide (2.08%), tri-pentanucleotide (1.04%) and penta-di-mononucleotide (1.04%) were also found (Fig. 3). These findings are in accordance with the results of [17,19]. The dinucleotide motifs showed a much larger polymorphism level than the trinucleotide, tetranucleotide and the other motifs classes. However, the number of repeat motifs does not affect the level of polymorphism rate. Among the most frequent motif viz., dinucleotide motifs, (GA)n repeats were more frequent (39.34%), followed by (CT)n with 27.87% and least by (TA)n with 1.64%. Among the trinucleotide motifs (CCT)n repeats were maximum. (ATAG)n repeats characterized 30% of the tetranucleotide motifs followed by (AGAT)n 20% whereas the other repeat classes (CATC)n, (GAGT)n, (GATG)n, (TAAT)n, (TCTT)n each represented 10%. On the

other hand, the number of repeats ranged from 5 to 46 for the different polymorphic microsatellite markers (Table 3). The findings of the physical position, type of repeat motif and no. of repeats of the polymorphic SSR markers in the present investigation will aid in formation of set of polymorphic SSR's which can further be used in genotyping of mapping population for QTL mapping and marker-assisted selection in breeding.

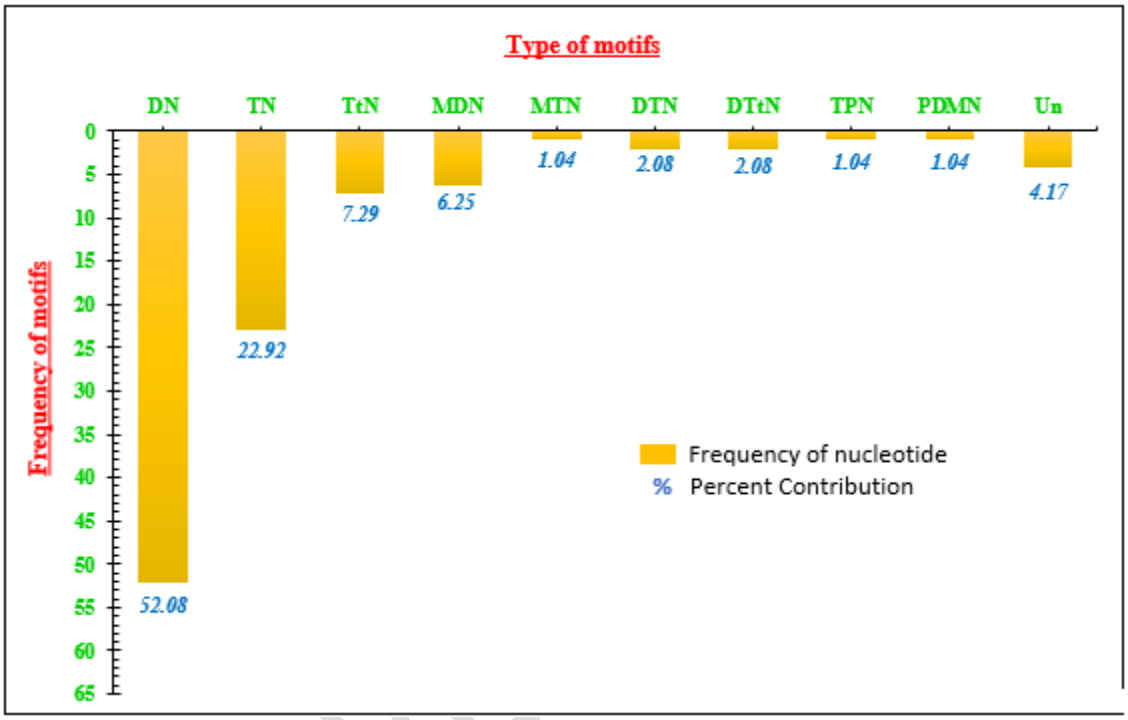

Fig. 3. Frequency distribution of different types of SSR repeat motifs. (DN: Dinucleotide, TN: Trinucleotide, TtN: Tetranucleotide, MDN: Mono-Dinucleotide, MTN: Mono-Trinucleotide, DTN: Di-Trinucleotide, DTtN: Di-Tetranucleotide, TPN: Tri-Pentanucleotide, PDMN: Penta-Di-Mononucleotide, Un: Unknown)

CONCLUSION:

Thus, in the present study, a total of 96 SSR markers including 4 gene specific markers were found to be polymorphic among the two parents indicating a total polymorphism percentage of 16.67%. Dinucleotide repeats were in much larger number as compared with other classes of nucleotides. The suitable polymorphic markers depending upon the nucleotide repeats will further be selected and utilized in genotyping of $F_{2:3}$ population and identification of QTLs for grain quality and yield traits.

Table 3: Identified polymorphic markers between the two parents along with their complete details is mentioned in table below:

Sr. no.	Name	Chr no.	Temp	Forward sequence	Reverse sequence	Map Distance	Motif	No. of repeats
1.	RM3252	1	57.4	GGTAACTTTGTTCCCATGCC	GGTCAATCATGCATGCAAGC	0.3	CT	13
2.	RM220	1	53.1	GGAAGGTAACTGTTTCCAAC	GAAATGCTTCCCACATGTCT	4.42	CT	17
3.	RM259	1	56.6	TGGAGTTTGAGAGGAGGG	CTTGTTGCATGGTGCCATGT	7.44	CT	17
4.	RM562	1	55.3	CACAACCCACAAACAGCAAG	CTTCCCCCAAAGTTTTAGCC	14.62	AAG	13
5.	RM24	1	58.2	GAAGTGTGATCACTGTAACC	TACAGTGGACGGCGAAGTCG	18.97	GA	29
6.	RM9	1	58.2	GGTGCCATTGTCGTCCTC	ACGGCCCTCATCACCTTC	23.32	GA, GT, GA	15, 1, 2
7.	RM543	1	59.3	CTGCTGCAGACTCTACTGCG	AAATATTACCCATCCCCCCC	32.78	GCG	10
8.	RM7180	1	48.3	GTGTTTATAGGGGTGCCACG	TGTTGGTGGTGCAGGTAAAG	34.1	ATAG	6
9.	RM1339	1	56.3	ATCAAAGCATGTAAACCAGC	CGTAAGATCTCCCTACCACC	38.19	AG	22
10.	RM14	1	55.7	CCGAGGAGAGGAGTTCGAC	GTGCCAATTTCCTCGAAAAA	41.36	GA	18
11.	RM279	2	57.3	GCGGGAGAGGGATCTCCT	GGCTAGGAGTTAACCTCGCG	2.88	GA	16
12.	RM71	2	58.3	CTAGAGGCGAAAACGAGATG	GGGTGGGCGAGGTAATAATG	8.76	ATT, T, ATT	10, 1, 4
13.	RM452	2	58.3	CGATCGAGAGCGTTAAGGG	GGGATCAAACCACGTTTCTG	9.56	GTC	9
14.	RM324	2	57.3	CTGATTCCACACACTTGTGC	GATTCCACGTCAGGATCTTC	11.38	CAT	21
15.	RM263	2	60.4	CCCAGGCTAGCTCATGAACC	GCTACGTTTGAGCTACCACG	25.86	CT	34
16.	RM526	2	57.5	CCCAAGCAATACGTCCCTAG	ACCTGGTCATGACAAGGAGG	26.66	TAAT	5
17.	RM14220	2	61.5	TCTCCACACAAACTTGGAACACG	GTCTGTATTGTGGGTGCAAGAGG	35.67	AC	14
18.	CHR3_14	3	52.6	GCCTACTCGTCTACCAACAA	GGCACTTATCCATTTCCAG	4.97	AT	n/a
19.	CHR3_20	3	55.3	ATTGACACGAAGAGGACAAA	GTGCCCGAGGTGAGTGAGT	5.95	GTA	n/a
20.	RM36	3	58.3	CAACTATGCACCATTGTCGC	GTACTCCACAAGACCGTACC	7.12	GA	23
21.	RM16	3	57.4	CGCTAGGGCAGCATCTAAA	AACACAGCAGGTACGCGC	23.12	TCG, GA	5, 16
22.	RM3513	3	61.5	TACTCCTATCCTGCCATGGC	TGTAGTAGACGAGAGGCCGG	25.11	CT	28
23.	RM4796	3	57.4	CCACGGTAGTTTTGGTCTAC	AGAGGGGAAGAGTGAGAGAG	27.47	TA	26
24.	RM520	3	55.3	AGGAGCAAGAAAAGTTCCCC	GCCAATGTGTGACGCAATAG	30.91	AG	10

25.	RM514	3	56.3	AGATTGATCTCCCATTCCCC	CACGAGCATATTACTAGTGG	35.28	AC	12
26.	RM335	4	59.8	GTACACACCCACATCGAGAAG	GCTCTATGCGAGTATCCATGG	0.68	CTT	25
27.	RM261	4	58.3	CTACTTCTCCCCTTGTGTCG	TGTACCATCGCCAAATCTCC	6.57	C, CT	9, 8
28.	RM1142	4	57.1	AAGCACACGTAAAACGGAGG	CGTCACTCTCACCACCACC	21.39	AG	12
29.	RM1388	4	54	TTCAATGAGGCAAAGGTAAG	ATTGTAGCTTGGACTAGGGG	25.2	AG	46
30.	RM317	4	59.8	CATACTTACCAGTTCACCGCC	CTGGAGAGTGTCAGCTAGTTGA	29.06	GC, GT	4, 18
31.	RM6748	4	52.7	ATTGGGTTTCTCATATTATG	CCAACACTCCTAACTAGTTC	30.9	TAT	18
32.	RM3843	4	61.5	ACCCTACTCCCAACAGTCCC	GGGGTCGTACGCTCATGTC	31.49	GA	23
33.	RM1113	4	55.3	GGGCGCATGTGTATTTCTTC	TGGGGAAAAACCACAAGCC	34.08	AG	12
34.	RM280	4	57.4	ACACGATCCACTTTGCGC	TGTGTCTTGAGCAGCCAGG	34.98	GA	16
35.	RM122	5	61.5	GAGTCGATGTAATGTCATCAGTGC	GAAGGAGGTATCGCTTTGTTGGAC	0.31	GA, A, GA, A, GA	7, 1, 2, 1, 11
36.	RM13	5	56.6	TCCAACATGGCAAGAGAGAG	GGTGGCATTCGATTCCAG	2.01	GA	16
37.	RM548	5	56.4	TCGGTGAGAAACTGAGAGTACG	AAGGAGGCCATCTCAATGTG	2.81	CT	12
38.	RM267	5	56.8	TGCAGACATAGAGAAGGAAGTG	AGCAACAGCACAACTTGATG	2.88	GA	21
39.	RM289	5	56.6	TTCCATGGCACACAAGCC	CTGTGCACGAACTTCCAAAG	7.8	G, GA	11, 16
40.	RM430	5	55	AAACAACGACGTCCCTGATC	GTGCCTCCGTGGTTATGAAC	18.69	GA	25
41.	RM163	5	60.4	ATCCATGTGCGCCTTTATGAGGA	CGCTACCTCCTTCACTTACTAGT	19.18	GGAGA, GA, C, GA	4, 11, 1, 20
42.	RM3616	5	56.3	GTGCGGATTTCTCCTCTCTC	TGCCGGTCCATTTCTAGAAG	26.28	GA	13
43.	SSI	6	57.4	GATCCGTTTTTGCTGTGCCC	CCTCCTCTCCGCCGATCCTG	3.09	n/a	
44.	RM217	6	54.4	ATCGCAGCAATGCCTCGT	GGGTGTGAACAAAGACAC	4.23	CT	20
45.	RM6836	6	53.9	TGTTGCATATGGTGCTATTTGA	GATACGGCTTCTAGGCCAAA	9.3	TCT	14
46.	RM564	6	54.3	TCCTTCTGCATCAATTCCTCTCG	CTCCATAGCCTTGTTAAGTGATGAGC	11.61	GT	14
47.	RM3183	6	56.6	GCTCCACAGAAAAGCAAAGC	TGCAACAGTAGCTGTAGCCG	12.44	CT	12
48.	RM1340	6	56.3	TCCAAACTAGTGGGAACGC	CTCAACGCCATGAACCTC	23.34	AG	22
49.	RM528	6	57.3	GGCATCCAATTTTACCCCTC	AAATGGAGCATGGAGGTCAC	26.55	AGAT	9
50.	RM30	6	59.8	GGTTAGGCATCGTCACGG	TCACCTCACCACACGACACG	26.87	AG, A, GA	9, 1, 12
51.	RM6965	7	53.2	TCATTTGGATCATAAGCTGG	TTGGATGAGATAACCAATGC	2.8	TTC	11

52.	RM481	7	57.3	TAGCTAGCCGATTGAATGGC	CTCCACCTCCTATGTTGTTG	2.88	CAA	12
53.	RM542	7	55	TGAATCAAGCCCCTCACTAC	CTGCAACGAGTAAGGCAGAG	12.71	CT	22
54.	RM432	7	55.5	TTCTGTCTCACGCTGGATTG	AGCTGCGTACGTGATGAATG	18.95	CATC	9
55.	RM351	7	58.2	CCATCCTCCACCGCCTCTCG	TGGAGGAAGGAAAGGGGACG	23.92	CCG, CGAAG	9, 4
56.	RM1132	7	61.5	ATCACCTGAGAAACATCCGG	CTCCTCCCACGTCAAGGTC	23.98	AG	12
57.	RM234	7	55	ACAGTATCCAAGGCCCTGG	CACGTGAGACAAAGACGGAG	25.47	CT	25
58.	RM3555	7	57.4	TGGAAGTTTCCTGGCGATAG	TGGTTGGACTGAAAAGTCCC	27.89	GA	12
59.	RM408	8	61.5	CAACGAGCTAACTTCCGTCC	ACTGCTACTTGGGTAGCTGACC	0.12	CT	13
60.	RM6863	8	54.4	GCTGCAGAATTAAGGAGAAC	TGCTCAAAATAATCAGCTCC	2.01	TGC	9
61.	RM5647	8	58	ACTCCGACTGCAGTTTTTGC	AACTTGGTCGTGGACAGTGC	2.89	AAG	16
62.	RM547	8	58.5	TAGGTTGGCAGACCTTTTCG	GTCAAGATCATCCTCGTAGCG	5.59	ATT	19
63.	CHR8_4	8	56.9	GATTGAAAGAGAAAGGTGGTT	CTGTGTAACCGAGTTACGTTT	9.95	ATAG	n/a
64.	RM404	8	57.3	CCAATCATTAACCCCTGAGC	GCCTTCATGCTTCAGAAGAC	15.43	GA	33
65.	RM42	8	58.3	ATCCTACCGCTGACCATGAG	TTTGGTCTACGTGGCGTACA	20.09	AG, AG, T, GA	6-2, 1, 5
66.	ARO7	8	54.4	ATTTGCCTCCTGAGTCTG	GAGGATGGGGAAGATAAA	20.26	n/a	
67.	BADEX7-5	8	53.1	TGTTTTCTGTTAGGTTGCATT	ATCCACAGAAATTTGGAAAC	20.32	n/a	
68.	nksbad2	8	61	GGTTGCATTTACTGGGAGTTATG	TCCACAGAAATTTGGAAACAAAC	20.38	n/a	
69.	RM80	8	57.7	TTGAAGGCGCTGAAGGAG	CATCAACCTCGTCTTCACCG	24.47	TCT	25
70.	RM316	9	56.7	CTAGTTGGGCATACGATGGC	ACGCTTATATGTTACGTCAAC	1.1	GT, TG, TTTG, TG	8-9, 4, 4
71.	RM5688	9	56.3	GCAGTGTCCAACCATCTGTG	ATCTGGTCACCCTTTGCTTG	1.71	AAT	17
72.	RM219	9	54.6	CGTCGGATGATGTAAAGCCT	CATATCGGCATTCGCCTG	7.9	CT	17
73.	RM7387	9	54.6	GCAGTAGGGAGCATGGAAAG	AAACGAGTCCTCTTCAGGGG	10.13	GAGT	6
74.	RM105	9	67.7	GTCGTCGACCCATCGGAGCCAC	TGGTCGAGGTGGGGATCGGGTC	12.6	CCT	6
75.	RM328	9	57.5	CATAGTGGAGTATGCAGCTGC	CCTTCTCCCAGTCGTATCTG	19.72	CAT	5
76.	RM160	9	66.7	CCCAAATCAGGAAAGTTTCTCAGC	AGTCATCCTTGGCTACCAGATGC	19.78	GAA	23
77.	RM3808	9	55.6	CGTTAGCGAAACGAACAGTG	CAGTGGCTCGGTAATCGC	20.54	GA	20
78.	RM474	10	55.3	AAGATGTACGGGTGGCATTC	TATGAGCTGGTGAGCAATGG	1.81	AT	13

79.	RM311	10	50.7	TGGTAGTATAGGTACTAAACAT	TCCTATACACATACAAACATAC	9.74	GT, GTAT, GT	3, 8, 5
80.	RM258	10	58	TGCTGTATGTAGCTCGCACC	TGGCCTTTAAAGCTGTCGC	18.01	GA, GGA	21, 3
81.	RM171	10	58.7	AACGCGAGGACACGTACTTAC	ACGAGATACGTACGCCTTTG	19.04	GATG	5
82.	RM590	10	58.5	CATCTCCGCTCTCCATGC	GGAGTTGGGGTCTTGTTCG	23.04	TCT	10
83.	RM286	11	56.9	GGCTTCATCTTTGGCGAC	CCGGATTCACGAGATAAACTC	0.38	GA	16
84.	RM202	11	55.6	CAGATTGGAGATGAAGTCCTCC	CCAGCAAGCATGTCAATGTA	9	CT	30
85.	RM229	11	57.4	CACTCACACGAACGACTGAC	CGCAGGTTCTTGTGAAATGT	18.4	TC, CT, C, CT	11, 5, 3, 5
86.	RM21	11	60.4	ACAGTATTCCGTAGGCACGG	GCTCCATGAGGGTGGTAGAG	19.6	GA	18
87.	RM206	11	56.3	CCCATGCGTTTAACTATTCCT	CGTTCCATCGATCCGTATGG	22.01	CT	21
88.	RM1233	11	53.2	TTCGTTTTCCTTGGTTAGTG	ATTGGCTCCTGAAGAAGG	26.53	AG	15
89.	RM5926	11	54.4	ATATACTGTAGGTCCATCCA	AGATAGTATAGCGTAGCAGC	28.43	ATT	21
90.	RM20	12	57.8	ATCTTGTCCCTGCAGGTCAT	GAAACAGAGGCACATTTCATTG	0.97	ATT	14
91.	RM19	12	54.4	CAAAAACAGAGCAGATGAC	CTCAAGATGGACGCCAAGA	2.43	ATC	10
92.	RM1337	12	55.3	GTGCAATGCTGAGGAGTATC	CTGAGAATCTGGAGTGCTTG	11.93	AG	21
93.	RM28305	12	56.5	GTCATCTTCGCAAATGGTGATGG	GGTCGTCGTGGTGTTATTCTTGG	19.92	GA	31
94.	RM28367	12	62	CGTATCTCCACCTCCCGAGAAGC	GCCAAATCTCACGGATCGAAGC	21.17	AG	21
95.	RM28556	12	61.1	CTAGTAGTGCCACTTAACCGTGTTCG	GGATCCAAACACCACCTTAGCC	23.98	TC	14
96.	RM17	12	59.3	TGCCCTGTTATTTTCTTCTCTC	GGTGATCCTTTCCCATTTCA	26.95	GA	21

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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