# Original Research Article

# Study ofParental Polymorphismand Allelic Variation for Grain Quality and Yield Traits in Rice (*Oryza Sativa* L.)using SSR Markers

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**ABSTRACT:**

**Aim:**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.

**Place and Duration of Study:**It was carried out at Molecular Breeding Lab, Department of Genetics and Plant Breeding, Institute of Agricultural Sciences,Banaras Hindu University,Varanasi - 221 005, India, during 2019

**Methodology:** Two parents Improved Samba Mahsuri and Badshabhog wereused for the present study. The DNA extraction was done as per the CTAB method suggested by Murray and Thompson. Standard PCR protocol was followed.

**Results:**For this a total of 576 randomly selected microsatellite SSR 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%). The gene specific markers nksbad2, ARO7, BADEX7_5 and SSI were found to be polymorphic.

Conclusion:Based on the present study it may be concluded that thepolymorphic markers identified 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 two third of the South East Asian population [24].In 2019, global rice production was755.47 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. Asiancountries 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 theever-increasing demand, several new varieties are breed every year to achieve thehighest yield along with resistanceto various biotic and abiotic stresses. 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].Moreover, the preference and taste varies 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 the 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 inheritanceand 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 QTLs for grain quality traits in rice is animportant forward genetic approach and using these dissected complex regionsin MAS 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 the 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 SSR 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 one percent [12]. Also, they can be used in populationstructure analysis, gene mapping and tagging,linkage map construction, tracing marker-trait association, Marker Assisted Selection (MAS) and others. [23]. So, developing rice varieties with desired grain quality *viz.,* aroma, nutritional, cooking and eating quality with high yield as well as resistance to various abiotic and biotic stresses by suing molecular techniques and breeding tools will help to alleviate several malnutritionalproblem,as well as will help farmers and food industries personnel to fetch high price of their produce.. 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 parentsfor desirable grain quality and yield traits.The first parent isshort 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 are 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 the eastern plain zone. Twenty-one days old seedlings of Improved Samba Mahsuri and donor Badshabhog were transplanted in the 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:**

| Sl.no | Trait name | Recipient | Donor |
|---|---|---|---|
| 1 | Name | Improved Samba Mahsuri | Badshabhog |
| 2 | Type | Released variety in 2008 jointly by ICAR-IIRR and CSIR-CCMB Hyderabad, India | Local landrace collected by Institute of Agricultural Sciences, BHU, Varanasi, U.P, India |
| 3 | Parentage | Samba Mahsuri*4/SS1113 | Local landrace |
| 4 | Yield | 4.75-5.0 tonnesha$^{-1}$ | 2.5-3 tonnesha$^{-1}$ |
| 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 by using extraction buffer (10% CTAB, 1M 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 estimatedon0.8 % agarose gel 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 SSR markers including 26 gene specific markers distributed on f 12 chromosomes of rice were used to identify polymorphism between Improved Samba Mahsuri and Badshabhog. This was confirmed and tested by using graphical genotypes (GGTs) software package 2.0.[29]. GGT 2.0 is particularly a plant-breeding software package that helps to visualize data of markers with known map positions on a genetic map and displays estimated lengths of genomic compositions and distribution of polymorphic markers across the length of chromosome according to their physical positions (Mb) as colored chromosome bar segments. The information regarding chromosomal location, sequences of primers (forward and reverse), physical position (SSR start and SSR end) and 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). 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 using GGT software.

### 2.4 PCR amplification:

PCR was carried out 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 for45 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 minand hold at 4°C for ∞. After completion of amplification, PCR products were stored at -20°C

### 2.5 Agarose gel electrophoresis:

The amplified products were resolved on 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 DocTM XR+, BIO-RAD, USA) and the banding pattern was observed and recorded for further analysis.

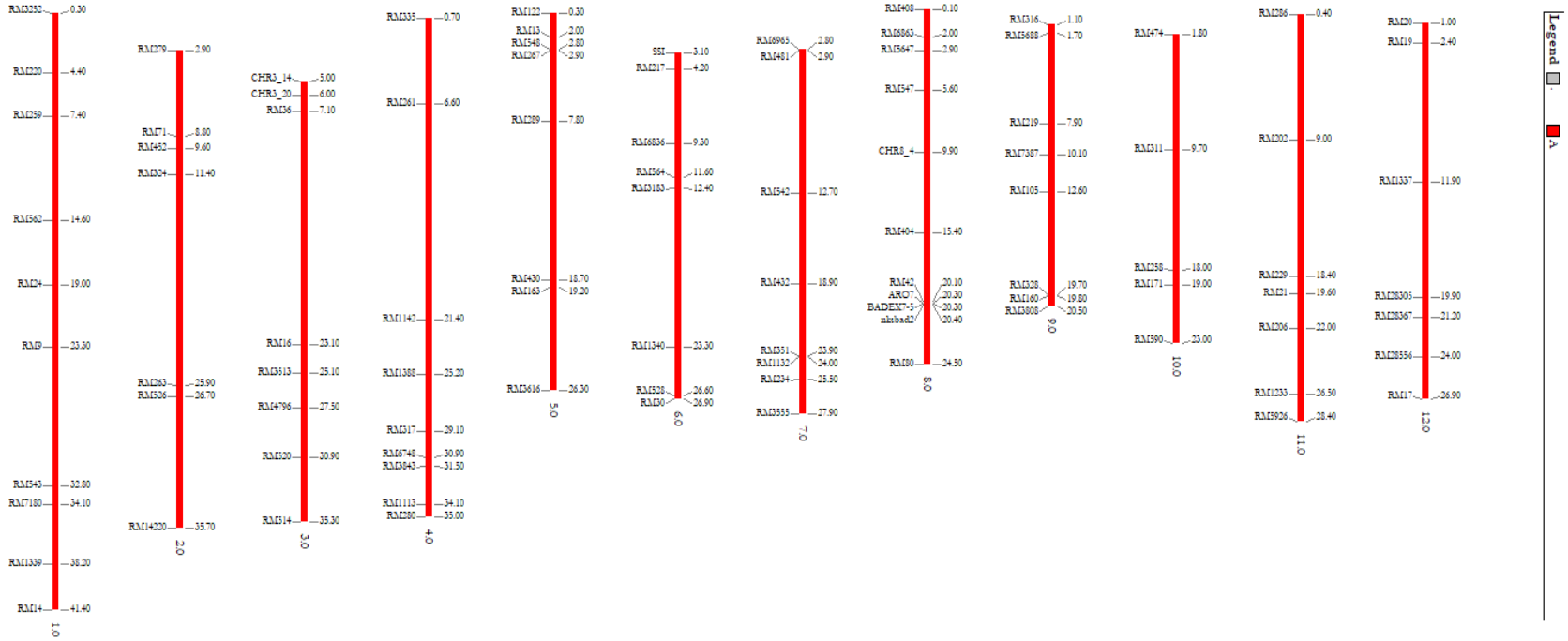
## 3. RESULTS AND DISCUSSION:

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. Amongthe 576 SSR markers used, 96 markers including 4 genespecific markers were found to be polymorphic between the two genotypes indicating a total polymorphism percentage of 16.67%. the polymorphic banding pattern of some of the markers are shownin gel picture Fig.2. 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%). 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 both the parents.

**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.**



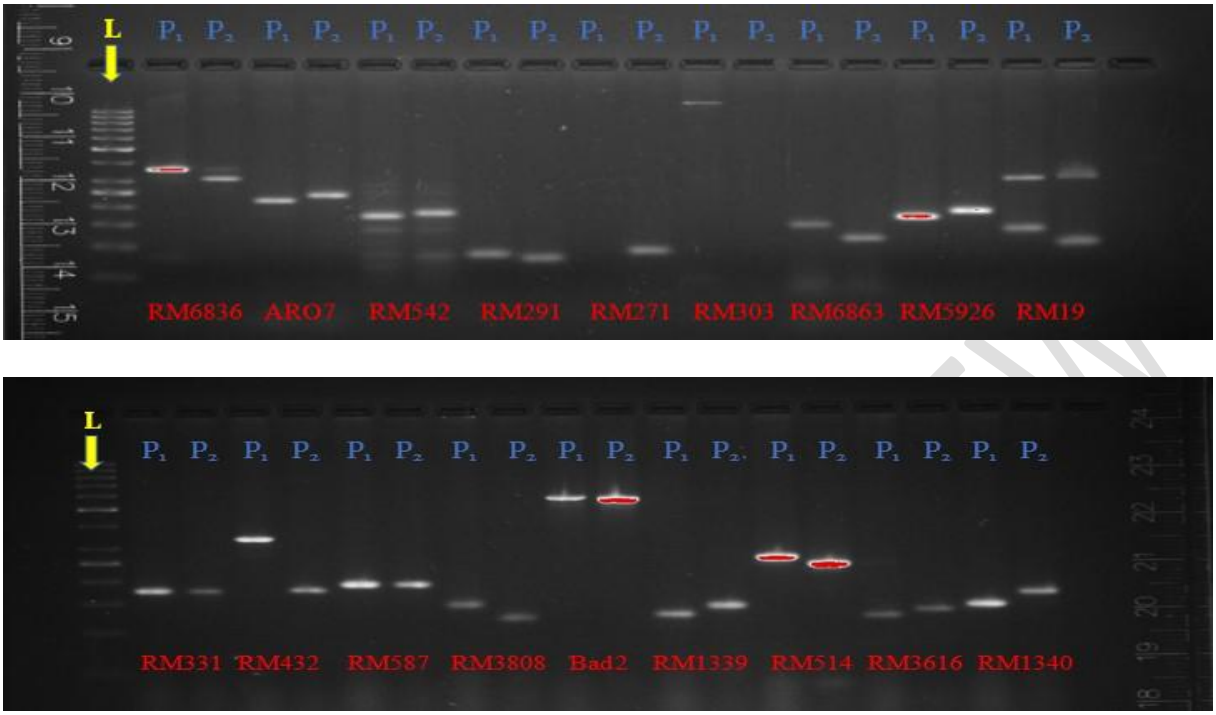



**Fig. 2. Gel picture showing parental polymorphism survey with SSR markers among the parents. P1- Improved Samba Mahsuri, P2- Badshabhog, L- Ladder 50 bp.**

The recent past studies on parental polymorphism also revealedcomparable results. The polymorphism percentage highest for chromosome 6 and lowest on chromosome 10 was also reported byShivani*et al.,*[23]. Similarly, Hable*et al.,*[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%).Chandu*et al.,*[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 Kulkarni*et al.,*[14] using 1,904 genomic SSRsmarkersamong the parents IR58025A and KMR-3R.

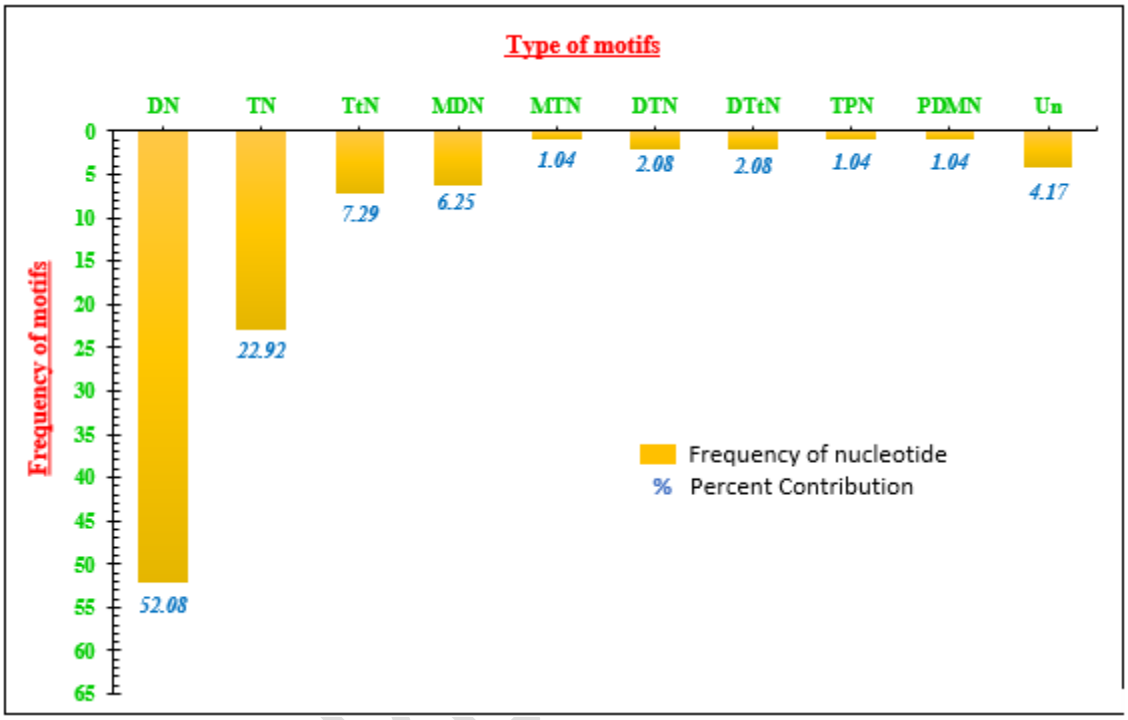
In the present study a total of 96 polymorphic markers were identified, out of which 20 SSR markers were reported to be associated with QTLs for different quality traits and the 4 gene specific markers were associated with genes of aroma and eating and cooking quality traits of rice from previous studies.Amongthe 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, BADEX 7_5 and nksbad2 is linked with BAD2 or badh2 genewith 8-bp deletion in the exon 7 which leads toaroma in rice. The gene specific primer SSIis 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 cookingBazrkar-Khatibani*et al.,*[5]. Xia*et al.,*[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.Dai*et al.,*[10] reported that RM7180, RM3616, RM6836, RM3183were 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 Singh*et al.,*[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 byVemireddy*et al.,*[30].Amarawathi*et al.,*[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 Cheng*et al.,* [8]. While Swamy*et al.,*[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 ofNarshimulu*et al.,* and Nicot*et al.,*[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 polymorphicSSR 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,it may be concluded that 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.Thesuitable polymorphic markers depending upon the nucleotide repeats will further be selected andutilized in genotyping of $F_{2:3}$ population and identification of QTLs for grain quality and yield traitsresults in development of improve rice cultivars having a more economic value.

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**Authors' contributions:**

This work was carried out in collaboration among all authors. Author SR and PK designed the study, performed the molecular work and wrote the first draft of the manuscript. Author PKS designed and supervised the study, proof read and edited of manuscript. Authors RK, SU, PR, BP, SSprovided inputs on designingthe study and interpretation of results.All authors read and approved the final manuscript.

**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 | CCCATGCGTTTAACTATTCT | 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.**

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