J. Agric. Res. Technol., 50 (2) : 215-220 (2025) https://doi.org/10.56228/JART.2025.50213

Study of Segregation Distortion in Segregating Lines of Rice using Molecular Markers

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(Received : 10.09.2024 Accepted : 07.04.2025)

Abstract

Rice (*Oryza sativa* L.) is a vital staple crop grown across diverse regions in Asia. This study focuses on screening F_2 segregating lines using molecular markers associated with amylose content. Amylose production is regulated by the Waxy (*Wx*) gene, which encodes Granule-Bound Starch Synthase I (GBSSI), with amylose levels directly linked to GBSSI in the endosperm. A breeding program was initiated to improve popular variety Indrayani for amylose content by crossing it with variety Bhogavati having high amylose content. The F_1 progeny of the cross was confirmed with QTL-linked markers and the F_1s were selfed to develop an F_2 population during *kharif* 2022. Significant segregation distortions were observed for 8 polymorphic SSR markers, with all markers showing distortion. Three distorted markers W2R, RM 8225, and RM 340 deviated significantly towards Indrayani, while remaining five markers Wx 484, RM 3, RM 8261, RM 217, and RM 190 were deviated towards Bhogavati. The results from the molecular markers revealed that only two F_2 plants, exhibited heterozygosity. These plants hold special significance and can be advanced to help identify desirable lines with improved amylose content in future generations.

Key words : Waxy gene, F_2 population, segregation distortion, amylose content.

In India, rice is one of the most important staple food crops, and supply food for over half of the population. India produced 137.82 million tonnes of rice on an area of 47.83 million ha with productivity of 2.88 t/ha milled rice during 2023 (https://www.fao.org/faostat/en/#data/QCL). In rice, amylose content (AC), gelatinization temperature, and gel consistencyare the three key factors which determine the cooking and the eating quality. Amylose content plays a crucial role in classifying rice varieties, as it directly impacts the texture and retrogradation of the cooked grains. These grain quality traits show continuous variation are are controlled by quantitative trait loci (QTLs). Use of molecular markers can provide the viable alternative to study the genetic basis of these quantitative traits. The Waxy (Wx) gene encodes a granule-bound starch synthase I (GBSSI), a protein that regulates AC and the viscoelasticity of cooked rice. The two alleles which leads to differential levels of AC are Wxa and Wxb in indica and japonica rice, respectively (Pathak *et* al. 2015).

Segregation distortion refers to the deviation of observed genotypic frequencies from the expected Mendelian segregation ratios (Manjappa *et* al. 2022). Several factors contribute to segregation distortion, including the mapping population, genetic transmission, selection at the gametic and zygotic stages, non-homologous recombination, gene transfer, transposable elements, and environmental influences (Xu *et* al. 1997; Knox and Ellis, 2002). Since DNA markers are not influenced by environmental factors, they are ideal for analyzing segregation distortion.

In this study, segregation distortion was observed in an F_2 population, which was

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analyzed to identify lines with improved amylose content. SSR marker analysis was employed to examine allele frequencies and genotype distribution, helping to identify factors that influenced the observed segregation patterns.

Materials and methods

Experimental material : A cross was made between varieties Indrayani and Bhogawati at Agricultural Research Station Radhanagari, District Kolhapur, MS (16°24'50" N latitude and 73°59'52" E longitude) with an objective to develop lines with improved amylose content in the genetic background of variety Indrayani. Indrayani is a popular variety grown in the Western Maharashtra with intermediate amylose content (17-18%), long slender, scented, translucent, midlate and moderately resistant to blast and blight. Bhogawati is having high amylose content (24-25%), medium height, late duration, long slender grain, scented, moderately resistant to blast and blight. The plant material used in this study comprised of F_2 population which was developed by selfing of the F_1 from this cross.

Molecular markers : The Waxy (*Wx*) gene is a key regulator of amylose content in rice, and selecting SSR markers associated with this gene is crucial for accurate screening. We chose the Wx 484 and RM 8225 SSR markers for confirmation of F1s. These markers have been used in previous studies (Mikami et al. 2008) for their reliable association with the gene, ensuring precise identification of desirable alleles related to amylose content. The F_1s which were heterozygous were selected for development of F_2 population.

For molecular marker analysis of F_2 population, 27 SSR markers earlier reported to be linked with amylose content, aroma and fragrance were used to screen the parental genotypes. These markers were carefully chosen for their linkage to relevant genes, genetic coverage, and reports from the previous studies. These markers represented Chromosomes 3, 5, 6, 7, 8, 10 and 11. Out of 27 markers used, 16 were from Chromosome 6, which harbours the genes/QTLs linked with the AC.

DNA isolation and SSR marker analysis : F_2 plants were grown in the field and leaf samples were utilized for genomic DNA extraction. Fresh leaves were collected from each F_2 plant and the parental lines and ground in liquid nitrogen. DNA was extracted from the ground tissue by the cetyl trimethyl ammonium bromide (CTAB) method as described earlier (Gavhane et al. 2019).

PCR reactions were carried out in sterile 0.2 ml thin-walled PCR tubes. Amplification was carried out in 20 µl reaction volume as per Kumbhar et al. (2013). Briefly, 20 ng DNA template, 200 µM of each dNTPs, 1 U μl^{-1} Taq DNA polymerase, 1X PCR reaction buffer, 0.1 µM of each primer and volume made to 20 µl. The PCR amplification was done in a thermo-cycler which was programmed as follow denatured at 94°C for 5 minutes followed by 35 cycles (30 sec denaturation at 94°C, 45 sec annealing at 55°C and 1 min of primer extension at 720C) of PCR amplification followed by final extension of 72°C for 5 minutes and the PCR products were resolved on 3.5% agarose gel treated with ethidium bromide stain to visualize bands under UV light by using Gel documentation system.

Scoring of data and interpretation : Being F_2 population, alleles were contributed by female parent Indrayani and by male parent Bhogawati. Therefore, allele type of F_2 plant similar to recurrent parent was recorded as '+' (positive) and similar to donor parent as '-' (negative), and heterozygous as '±' and missing allele as '.'. Since alleles in homozygous condition are stable over the generations only F_2

plants with heterozygous alleles were selected. Number of homozygous alleles (+) i.e., similar to recurrent parental line for desired traits in each of 100 F_2 plants were also recorded.

Analysis of segregation distortion of markers : In this research, amylose trait specific SSR markers were screened on the F_2 population. Since lot of segregation distortion was obtained, chi-square test was employed to test the segregation distortion of SSR marker from the normal Mendelian (1:2:1 or 3:1) ratio in F_2 population. Additionally, chi-square test was used to compute segregation distortion by biparents' genotypes to ascertain whether they are skewed towards the female parent or the male parent.

For the codominant markers, allele frequency (p = q) and the distribution of different genotype frequencies in the F_2 population (p^2:2pq:q^2) were analyzed to characterize factors resulting in distorted segregation. The segregation ratio of marker genotypes was analyzed using the Chi-square test with the following formula:

$$\chi^2 = \sum_{i}^{p} (n_i - E_i)^2 / E_i$$

where, p : number of classes, n_i : observed number of units falling into class i and E_i : number of units expected to fall into class i

The null hypothesis of the test was that the markers segregate in a 1:2:1 or 3:1 ratio. The observed segregation ratio was declared not deviating from the expected ratio if the value of calculated χ^2 was less than the value of χ^2 table at α = 0.05 or 0.01 (Zhang *et al.* 1997).

Results and Discussion

Development of F_1 and F_2 plants : F_1 plants were confirmed for desirable allele by using polymorphic markers *Wx* 484 and RM 8225. By using these markers, we aimed to efficiently confirm the presence of favourable Wx alleles in the F1 generation, thus facilitating the selection of superior lines for further breeding. After confirmation, true F_1s were selected for selfing to develop F_2 population. Out of total 27 SSR markers screened, eight SSR markers found polymorphic between the parental genotypes were used to screen the F_2 population. One hundred F_2 plants were selected for this purpose. These F_2 individual plant selection (IPS) and parents viz., Indrayani and Bhogawati were evaluated using eight polymorphic markers.

Screening of F_2 population using molecular markers : It was observed that all the eight markers used for screening the F2 population showed distorted segregation (Table 1). For instance, marker Wx 484 amplified in 99 F2 plants, with 98 plants showing alleles resembling the donor parent and one plant (plant # 63) being heterozygous, while none of the plant showed alleles resembling to the female parent. From 100 F_2 plants amplified by the marker W_2R, 90 plants exhibited homozygous alleles i.e, similar to Indrayani, 9 resembled to Bhogavati, and 1 plant was heterozygous (plant # 63). Marker RM 3 amplified in 100 F2 plants, with 7 plants exhibiting alleles similar to Indrayani, while 92 plants amplified alleles similar to Bhogavati, and 1 plant was heterozygous (plant # 63). Marker RM 340 amplified in 94 F_2 plants, with 93 showing homozygous alleles corresponding to Indrayani, while one plant was heterozygous (plant # 87), and none of the plants resembled the donor parent.

Marker RM 217 amplified in 91 F_2 plants, all of which showed alleles similar to the donor parent. Marker RM 190 amplified in 96 F_2 plants, of which 85 plants resembled to Bhogawati, 10 resembled to Indrayani, and only one plant exhibited heterozygosity (plant # 63). Using marker RM 8225, 91 F_2 plants were

amplified. Among them, 90 plants amplified alleles similar to Indrayani, while 1 plant showed alleles resembling Bhogawati. Marker RM 8261 amplified in 99 F_2 plants, of which 11 showed alleles resembling Indrayani, 87 resembling Bhogawati, while one was heterozygous (plant # 63; Table 1). In order to rule out the possibility of concentration of agarose gel affecting resolution of the amplified product, the markers were screened with concentration of agarose at 3 to 3.5%.

Analysis of segregation distortion of SSR markers : The eight SSR markers which were used to screen the F_2 population showed distorted segregation compared to the expected Mendelian inheritance ratios (3:1), with a tendency to deviate towards the female or male parent. In this study, the expected Mendelian ratio for each marker in the F_2 populations was 3:1 (Table 1).

Chi-square test was used to assess segregation distortion, revealing two types of skewness: one favouring the recurrent parent, Indrayani, and the other favouring the donor parent Bhogawati. The markers showed consistent distortion towards either of the parental allele. For instance, markers W_2R, RM 8225, and RM 340 from chromosome 6 were skewed towards Indrayani, while *Wx* 484, RM 3, RM 8261, RM 217, and RM 190 were skewed towards Bhogawati. Chi-square test performed on 8 co-dominant SSR markers revealed that all SSR markers (100%) were distorted from normal Mendelian segregation ratio (Table 1). Three markers W_2R, RM8225, and RM340 were distorted significantly towards female parent ($P<0.01$) and *Wx* 484, RM3, RM8261, RM217, and RM190 were distorted towards donor parent ($P<0.01$) (Table 1). The marker *Wx* 484, which was skewed towards Bhogawati with a $\chi 2$ value of 49.5, showed highest level of deviation among the distorted SSR markers while marker RM 190 showed lowest level of deviation among the distorted SSR markers towards Bhogawati with a $\chi 2$ value of 20.67. Except for one marker, the remaining seven markers which showed distortion were mapped on chromosome 6. Zhao *et* al. (2006) also reported distortion in several SSR markers located on this chromosome. They reported that 33.1% of markers in an F_2 population from a Nipponbare x Guangai 4 cross showed segregation distortion, while Peng et al. (2006) found 63% of markers distorted in a recombinant inbred line (RIL) population. These studies suggest that segregation distortion is common in inter-subspecific populations. The genetic backgrounds of the parents may contribute to this segregation distortion. In this

Table 1. The details of the Chi-square test utilized to evaluate the segregation distortion of the SSR marker in the F_2 population

Marker	Chromosome	Total plants amplified	Resemblance of genotype in the F_2 population			χ^2	Direction of skewness
			Indrayani	Heterozygous	Bhogawati		
Wx 484	6	99	00	1	98	49.50**	Bhogawati
W2R	6	100	90	1	09	26.64**	Indrayani
RM 3	6	100	07	1	92	31.21**	Bhogawati
RM 340	6	94	93	1	00	47.01**	Indrayani
RM 217	6	91	00	0	91	45.50**	Bhogawati
RM 190	6	96	10	1	85	20.67**	Bhogawati
RM 8225	6	91	90	0	01	42.55**	Indrayani
RM 8261	7	99	11	1	87	21.99**	Bhogawati

study, the F_2 population from the Indrayani x Bhogawati cross showed 100% segregation distortion, a higher rate than reported in previous studies.

This distortion is likely influenced by gametic selection, as noted by Manjappa et al. (2022). Segregation distortion regions (SDRs) on rice chromosomes are the regions where most distorted markers are clustered. Xu et al. (1997) reported that these regions are linked to 11 gametophyte (ga) genes and 20 sterility (S) genes. Of 17 SDRs identified, 8 overlap with gametophytic and sterility gene loci. The clustering of distorted markers suggests genetic factors, rather than errors, drive segregation distortion. Marker W2R, linked to the Waxy gene on chromosome 6, also resides in an SDR, highlighting the role of gametic and sterility genes in this distortion.

Identification of desirable segregants for amylose content : From the results obtained using molecular markers it was observed that only two F_2 plants showed heterozygous nature (plant # 63 and 87). These plants are of special importance and can be advanced to identify desirable lines for amylose content in the subsequent generation. Similarly, the F2 plants resembling to those of Indrayani can be advanced further. Since estimation of amylose is an invasive technique, the F_2 seeds cannot be used for amylose estimation. However, the desirable F_2 plants can be advanced further and estimation of amylose can be done in the $F_{2:3}$ progenies.

Conclusions

One hundred F_2 plants screened with 8 polymorphic SSR markers associated with amylose content indicated that the marker genotypic frequencies were deviating from the expected Mendelian ratio (1:2:1 or 3:1) in a segregating population. Through chi-square analysis of these markers, it was found that all markers showed significant distortion from Mendelian inheritance ratios. Among these markers, 5 markers (RM 190, Wx 484, RM 3, RM 217 and RM 8261) deviated towards donor parent (Bhogawati) and 3 markers (RM 8225, W2R and RM 340) towards female parent (Indrayani). The results from the molecular marker analysis revealed that only two F2 plants, (plant # 63 and 87) exhibited heterozygosity. These plants may be useful in identification of desirable segregants with higher amylose content in the subsequent generations.

Acknowledgements

PLK would like to thank Department of Biotechnology, Govt. of India for research support during this period.

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