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Rice hybrid purity testing at molecular level for development of F_2 mapping population

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Abstract
Microsatellite markers are most commonly use in fingerprinting, for assessing variation within parental lines and testing the genetic purity of hybrid seed in rice at molecular level. The present investigation was undertaken to assess the hybridity of experimental F_1 plants derived from the cross between two lines naming NVSR 2179 and NVSR 2803 having contrasting characters for yield and its attributes. Both of the parents screened using five SSR (Simple Sequence Repeat) markers to identify polymorphic marker. The SSR marker naming RM225 found to be polymorphic between two parents. Total 46 F_1s and parents were screened using polymorphic marker RM225 using PCR method. The results showed all the 46 plants are true hybrid at molecular level and can be used for development of mapping population. Screening of 100 individuals of F_2 mapping population using same SSR markers further conformed its purity base on mendalian segregation pattern of marker alleles.

Keywords: Rice, Hybridity assessment, SSR, Polymorphic markers.

1. Introduction
Rice (*Oryza sativa* L.) is the most important cereal crop of the world. Rice is a self-pollinated crop and diploid in nature with twelve pairs of chromosomes (2n = 24). Rice is princess among the cereals have twenty four recognized species belonging to genus *Oryza* and tribe Oryzeae in the family Poaceae (Gramineae). *Oryza sativa* L. and *Oryza glaberrima* Steud. are the only two cultivated species. *Oryza sativa* L. is believed to have originated in Asia in the region encompassing North-Eastern India, Northern Bangladesh, the triangle adjoining Burma, Thailand, Laos, Vietnam and Southern China. *Oryza glaberrima* Steud. is believed to have originated on the swampy upper Niger river basin in Africa. The morphology, physiology, agronomy, genetics and biochemistry of *O. sativa* have been intensely studied over a long time. More than 40,000 varieties of rice had been reported worldwide (Gopalan *et al* 2007) [11].
“Rice is life” was the famous theme of International Year of Rice, 2004 denoting its overwhelming importance as an item of food and commerce. Rice remains a staple food for about half of the global population grown in 162.56 million ha with 504.94 million tons (Mt) milled rice production (Anonymous, 2021) [2]. It provides more than one fifth of the calories consumed worldwide by humans. Over 2 billion peoples in Asia alone derive 80% of their energy needs from rice, which contains 80% carbohydrates, 7 - 8% protein, 3% fat and 3% fiber (Chaudhari *et al.*, 2018) [7]. According to USDA, 1 cup (186 g) of cooked white rice contains 242 K calories, fat: 0.4 g, carbohydrates: 53.2 g, fiber: 0.6 g, protein: 4.4 g. It is also a good source of thiamine, riboflavin, niacin and dietary fiber. Unmilled rice contains more nutrients than milled or polished white rice. It is one of the oldest and second most intensively grown cereal crop and ranks third in grain production.
Asia is considered to be the ‘rice bowl’ of the world, and it produces and consumes more than 90 percent of world rice. India is the largest rice growing country, while China is the largest producer of rice. Rice is the staple food of 65 percent of the total population in India. Rice constitutes about 42 per cent of the total food grain production and 45 per cent of total cereal production of our country. In India, rice is cultivated in 43.78 million hectares with production of 118.43 million tonnes and productivity of 2705 kg per hectare (Anonymous, 2020) [1, 3]. Chhattisgarh is known as “rice bowl” of India while, the cultivated area of rice in Gujarat is 9.06 lakh hectares along with production 21.47 lakh tonnes and productivity of 2369.7 kg/ha (Anonymous, 2021) [3].
Rice has been adopted as an important model system for monocot plant research, largely due to its small genome size (*indica*-390Mb and *japonica* 430Mb) among cereals (maize 3,300Mb,

barley 5,100Mb and wheat 16,000Mb (Ashikari and Matsuoka, 2005) [4], vast germplasm collection (>1,20,000 accession worldwide), enormous amount of genetic variability, availability of a high-quality reference genome sequence, shares substantial collinearity with members of the grass family, highly saturated molecular marker linkage maps and well established transformation system (Paterson *et al.* 2005 and Xing and Zhang, 2010) [17, 24].

Yield is a complex trait, inherited in a quantitative manner and typically controlled by a number of major and minor quantitative trait loci. Polygenes produce small individual effects on the trait phenotype, but the effects of all the polygenes affecting a given trait are cumulative. The chances of increasing yield by changing cultural practices are limited. Therefore, future improvements in yield of food crops may depend entirely on genetic improvements. The higher demand of rice production may be achieved by shifting from entire dependence on conventional hybridization and selection to merging of classical breeding techniques with modern plant biotechnological tools like molecular markers for crop improvement (Stuber *et al*., 1999) [19].

QTL mapping is a forward genetics approach and involves genetic dissection of genomic regions which are associated with complex phenotypic traits using proper statistical strategy and analysis of segregating material (Tiemey *et al*, 2005) [23]. Availability and purity of mapping populations like F_2, F_3, recombinant inbred lines (RILs), backcross, double haploids (DH) is one of the pre-requisite for QTL mapping. Microsatellite markers are most commonly use to assess genetic purity of hybrid seed in rice at molecular level to develop mapping population.

Materials And Methods

Plant material

Crossing program was conducted at Main Rice Research Station, Navsari Agricultural University, Navsari in summer, 2019 to get F_1s for development of F_2 mapping population. The molecular work was carried out at Molecular Laboratory, Genetics and Plant breeding Department, N. M. College of Agriculture, Navsari Agricultural University. The female parent naming NVSR 2179 is crossed with a male parent NVSR 2803 to develop F_1 seeds during summer 2019. The crossing work was started, when the crop commenced flowering, emasculation was done during evening hours followed by pollination on next day morning. The F_1 seeds of individual parental lines were harvested separately and were labeled accordingly. Total 50 F_1 seeds of a cross NVSR 2179 × NVSR 2803 were grown along with the parents in lowland condition at Main Rice Research Station, Navsari Agricultural University, Navsari in *Kharif,* 2019. The F_1 plants were confirmed for heterozygosity by phenotypic observation and 4 off type plants are rougout. The leaf samples are collected from parents and remaining 46 F_1s for DNA isolation for hybridity testing at molecular level. F_2 mapping population of 100 individuals were used to for further confirmation of purity of selected hybrid plant.

DNA Isolation and PCR amplification

The total genomic DNA was isolated from parents (NVSR 2179 and NVSR 2803), F_1 plants and individuals F_2 population (100) with the protocol of Doyle and Doyle, 1990 with some modifications. The extracted DNA content was quantified and parental polymorphism studies were carried out through 5 SSR primers (Table 1). PCR mix for one reaction (volume 20 µl) contained 2 µl DNA, nuclease free water 12 µl, 2.5 µl 1x taq buffer + $MgCl_2$, 1 µl dNTP, 1 µl of each forward and reverse primers, and 0.5 µl Taq DNA polymerase. PCR amplification was performed with the following steps: pre-denaturing at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 sec., 55 °C for 45 sec. and 72 °C for 1 min, and last step for 10 min at 72 °C. Amplified products were analyzed using 5% agarose gel. The gel along with the DNA sample was stained with green satin (5 µl/100 ml). Electrophoresis was carried out for 2 hr at 90 volts and visualized under UV-transilluminator.

Chi-Square Analysis

Chi-Square statistic and comparing it against a critical value from the Chi-Square distribution allows the researcher to assess whether the association seen between the variables in a particular sample is likely to represent an actual relationship between those variables in the population.

$$\chi^2 = \sum_i \frac{(Oi - Ei)^2}{Ei}$$

Where, Oi is the observed number of cases in category i , and Ei is the expected number of cases in category i. (Campbell, 1989) [6]. If the calculated values of $\chi 2$ is significant at 1 per cent level of significance, is said to be poor and one or more observed frequencies are not in accordance with the hypotheses assumed and vice versa. So, it is also known as goodness of fit. The degree of freedom (df) in $\chi 2$ test is (n-1). Where n = number of classes.

Results And Discussion

Determining the purity of hybrid seed is an essential requirement for its use in development of mapping population since there is always a chance of contamination in the hybrid seed production plot because of pollen shedders, out crossing and physical mixtures during the subsequent handling of the harvested material.

Two parental lines belongs to *Indica* group *viz.,* NVSR 2179 and NVSR 2803 were used for development of F_2 mapping population for QTL mapping. Total 112 seeds of F_1 hybrid successfully harvested from crossing programme. Out of 112 seeds 60 seeds were sown in the nursery bed and 50 seeds were germinated. These 50 seedlings were transplanted in the field and four doubtful plants were rouged out from field base on morphological characters. Leaf samples from remaining 46 plants and two parents were collected for DNA isolation and PCR amplification.

Total five SSR primers were used to detect the polymorphism between parents (Figure 1). Out of that RM225 found to be polymorphic between two parents. This primer used to screen 46 individuals of F_1 for hybrid purity testing and the results clearly showed that all the individuals having both the allele of male and female parent. In this manner banding pattern of hybrid plants and their parental lines were studied and found that pure hybrid gives two bands corresponds to their parental lines bands (Figure 2).

After conforming the purity of 46 F_1 hybrids one plant randomly selected for development of F_2 mapping population. Same primer RM225 was screened in a 100 individuals of F_2 mapping population which showed presence of 29 female like alleles, 54 heterozygous type alleles and 17 male like alleles (Figure 3). The Chi-square results ($\chi 2$ = 3.52) indicated the marker allele was non-significant when compared with table

Chi-square value at 2 degrees of freedom and 0.05 probability. Hence, our selected marker follows the mendalian segregation pattern (1:2:1) in the F_2 mapping population. It's further conformed that our selected F_1 individual was a true hybrid based on mendalian segregation pattern [1 homozygote from female parent, 2 heterozygote from both parents, 1 homozygote from male parent] of marker alleles (Table 2). Similarly, SSR markers have been used successfully for hybrid purity testing by Yang *et al.* 1994 [25], Sundaram *et al.* 2007 [20], Muhammad *et al.* 2009 [16], Tamilkumar *et al.* 2009 [21], Kumar *et al.* 2012 [13], Deshmukh *et al.* 2013 [8], Gimhani *et al.* 2014 [10], Bora *et al.* 2016 [5], Manohara *et al.* 2020 [15] and Kumar *et al.* 2021. Marker RM225 also previously reported by Tian *et al.* (2006) [22] and Kim *et al.* (2017) [12] on chromosome number 6.

Table 1: List of primers of molecular markers used for polymorphism study

Sr. No.	Marker	Forward (5' to 3')	Reverse (5' to 3')
1	RM3600	TGCCCACACATGATGAGC	AACGGGCAAGAGATCTTCTG
2	RM225	TGCCCATATGGTCTGGATG	GAAAGTGGATCAGGAAGGC
3	RM9	GGTGCCATTGTCGTCCTC	ACGGCCCTCATCACCTTC
4	RM5474	AAAGTGTTGGTGAGCATAGC	TTTGTGTTTGGAGAGACGAG
5	RM1	GCGAAAACACAATGCAAAAA	GCGTTGGTTGGACCTGAC

Source: - Gramene data base (Link- https://archive.gramene.org/)

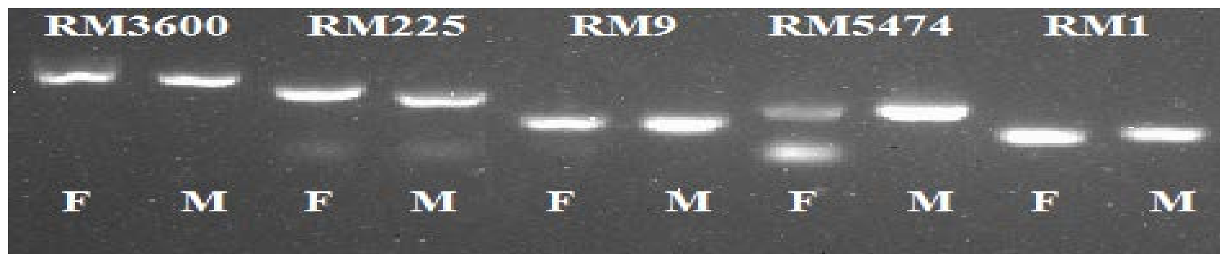

Fig 1: Polymorphism in NVSR 2179 (F) and NVSR 2803 (M) for five SSR markers

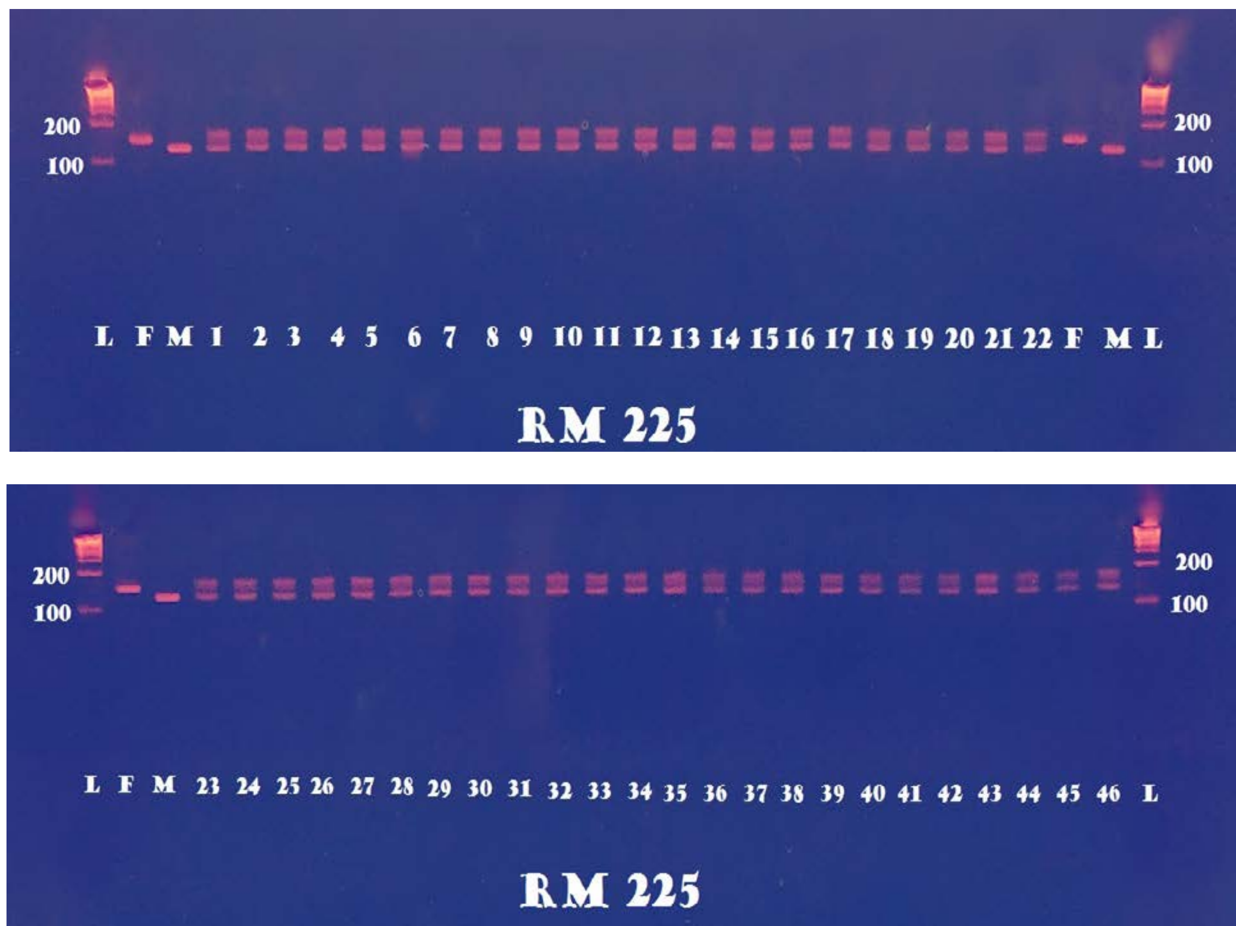

Fig 2: Hybridity testing in 46 F_1s of NVSR 2179 (F) × NVSR 2803 (M) using RM225 marker

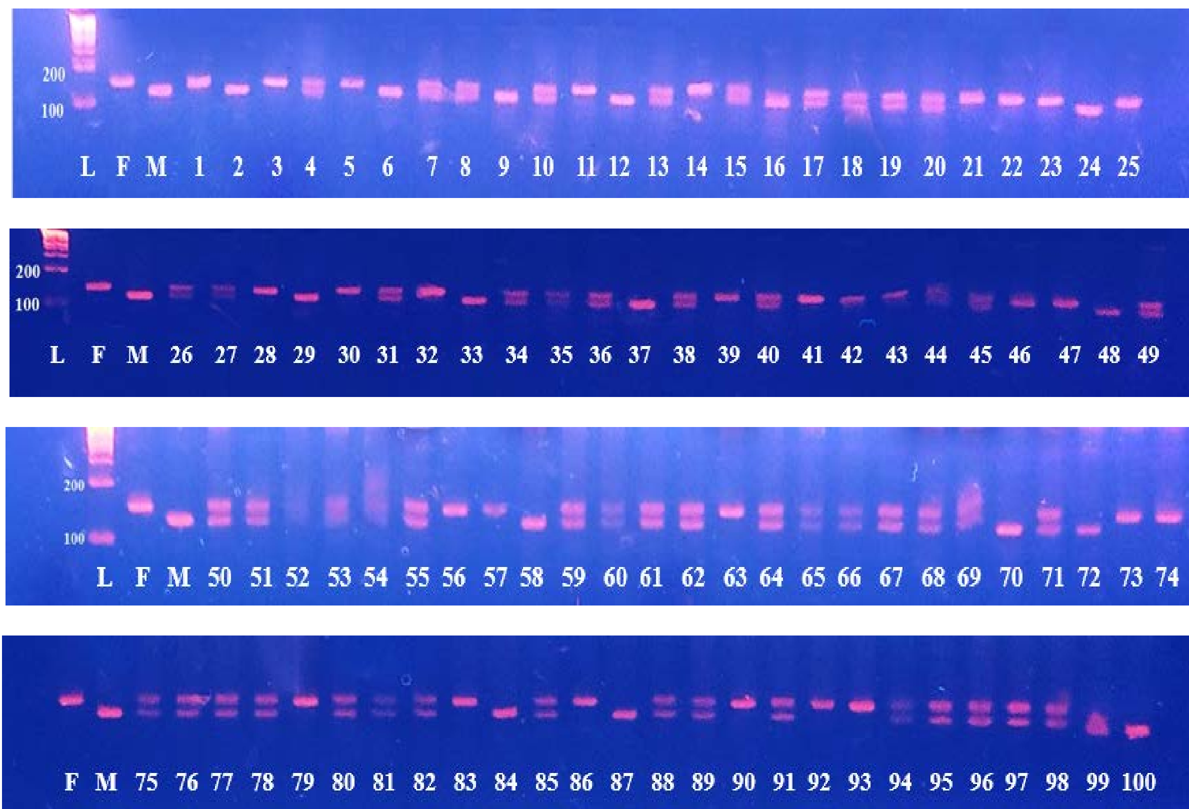

Fig 3: Segregation pattern of RM225 marker in F_2 mapping population (L: Ladder (100 bp), F: Female parent, M: Male parent)

Table 2: Segregation pattern and Chi-square tests for RM225 markers used to discriminate 100 individual F_2 plants

Sr. No.	Particulars	Details
1	Polymorphic marker	RM225
2	Chromosome number	6
3	Position on chromosome	21.1 cm
4	Female type allele (NVSR 2179)	29
5	Heterozygous allele (NVSR 2179 × NVSR 2803)	54
6	Male type allele (NVSR 2803)	17
7	Missing allele	0
8	Chi-square value	3.52

Note: Tested at 0.05 table value, * significant Chi-square value.

Conclusion

The results of present investigation revealed all the 46 plants are true hybrid at molecular level and can be used for development of F_2 mapping population for QTL mapping. Screening of 100 individuals of F_2 mapping population using same SSR markers further conformed its purity.

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