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MOLECULAR CHARACTERIZATION OF RICE (*Oryza sativa* L.) GENOTYPES USING SSR MARKERS ASSOCIATED WITH GRAIN IRON AND ZINC TRAITS

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ABSTRACT

Rice (*Oryza sativa* L.) is a major staple crop and an important target for micronutrient biofortification aimed at reducing dietary iron (Fe) and zinc (Zn) deficiencies. The present study assessed molecular diversity among 50 rice genotypes, including advanced Basmati breeding lines, Basmati and scented genotypes, and non-Basmati varieties, using 13 simple sequence repeat (SSR) markers. The marker set included candidate gene-based markers and genomic SSR markers previously reported in relation to iron and zinc associated genomic regions. Considerable molecular polymorphism was detected among the evaluated genotypes, with variation in the number of amplified fragments and polymorphism information content (PIC). Based on the values reported in the present study, PIC value ranged from 0.06 to 0.86, with RM152 showing the highest reported value. UPGMA-based cluster analysis separated the 50 rice genotypes into two major groups, demonstrating appreciable molecular diversity within the germplasm panel. The observed marker polymorphism may be useful for germplasm discrimination and selection of genetically diverse parental material. However, direct phenotypic validation of grain Fe and Zn concentrations would be required before individual markers or genotypes can be recommended specifically for micronutrient biofortification through marker-assisted breeding.

KEYWORDS: Biofortification, Genotypes, Micronutrient, Molecular markers, SSR

INTRODUCTION

Rice (*Oryza sativa* L.) is an important cereal crop and serve as a staple food for more than 50% of the world population. It is grown on 168.36 million hectares with an estimated production of 540.4 million metric tons of milled rice in 2025 (Statista, 2025). Besides playing a major role in food security, rice has a significant cultural, economic and dietary importance particularly in areas where its consumption is high. India is a major producer of rice, with an area of 52.83 million hectares and production of 154.02 million tonnes annually (Unified Portal for Agricultural Statistics (UPAg), 2025). Rice provides carbohydrates, protein, fat, fibre, and moisture, which are all essential to the diet. Its nutritional value is enhanced by the presence of vitamins and minerals, especially in the bran and germ layers of the rice grain, making it a staple food in diets around the world.

Brown rice is naturally rich in minerals but is often eaten polished, which diminishes the minerals. This refined rice lacks micronutrients and vitamins, which results in malnutrition in the case of rice being a staple food in developing areas. The amount of iron and zinc in polished rice is on average about 2 and 12 parts per million (ppm) respectively. These values fall considerably short of the nutritional benchmarks-13 ppm of iron and 28 ppm of zinc-recommended meeting 30% of the estimated average requirement (EAR) in daily diets (FAO Statistics, 2018). As a result, iron and zinc deficiencies affect over two billion people globally, with children and pregnant or lactating women being the most vulnerable groups (Young *et al*., 2023). Iron (Fe) and zinc (Zn) are vital nutrients necessary for proper growth and development in both plants and humans. Around one-third of the global population is affected by iron deficiency anemia (IDA), while zinc deficiency impacts nearly 20%, highlighting the pervasive issue of "hidden hunger" on a global scale. These nutrient deficiencies remain significant public health concerns, particularly in vulnerable regions (Garcia-Oliveira *et al*., 2018; United Nations Environment Programme, 2021).

Therefore, biofortification for micronutrients is very much in demand to maintain a good nutrient balance in our diet. Considering the severe problems of hidden hunger in many developing countries, several biofortification programmes have come into play to address this major issue. Improving the micronutrient contents in crop plants by conventional plant breeding and modern biotechnology methods to achieve sound health system in human being is called as biofortification (Pfeiffer and McClafferty, 2007). It increases bioavailability of micronutrient content in crop plants and thus helps in fighting the problem of nutrients deficiency. The Biofortification Priority Index has emphasized the critical need for iron (Fe) and zinc (Zn) biofortification in several Asian nations to combat prevalent micronutrient deficiencies. In response, the International Rice Research Institute (IRRI), in

collaboration with national research organizations in these countries, has made significant strides in developing rice varieties enriched with iron and zinc. To date, 29 high-zinc rice varieties have been developed and released for commercial cultivation across Bangladesh, India, Indonesia, and the Philippines. Notably, India has made remarkable progress in this initiative, contributing the highest number of these varieties, with 16 high-zinc rice lines officially released (Senguttuvel *et al*., 2023).

Both conventional and modern biotechnological methods have been proven to be very efficient in improving nutrient contents and it also shows that once variety with good balance of micronutrient is developed, it is long lasting over period with small expenditure in future breeding programmes (Gomez-Galera *et al*., 2010). Screening of germplasm lines for micronutrient contents is the preliminary method of conventional plant breeding. According to Graham *et al*. (1999), considerable genetic variation has been studied for iron and zinc content in germplasm accessions of rice. Genetic variation for iron and zinc content has been studied by many workers (Gregorio *et al*., 2000; Brar *et al*., 2011; Anuradha *et al*., 2012a).

Microsatellites, also known as Simple Sequence Repeats (SSRs), have emerged as one of the most extensively utilized markers in plant genotyping over the past two decades. Their popularity stems from several advantageous features: they exhibit codominance, provide high efficiency and reproducibility, are evenly distributed across the genome, and require only a small amount of DNA for analysis. Additionally, SSR markers are cost-effective and versatile, as they can often be applied to related crop species, enhancing their utility in breeding programs and genetic studies (Matin *et al*., 2012). Extensive research employing SSR markers has investigated the genetic diversity in rice and its relationship with micronutrient enrichment especially for iron and zinc (Chandu *et al*., 2020; Vanitha *et al*., 2018; Kumar *et al*.,2014; Stangoulis *et al*., 2007; Xu *et al*., 2015). Improving the levels of grain iron and zinc in staple crops through biofortification, using advanced molecular markers and breeding techniques, presents a sustainable solution to addressing micronutrient malnutrition in humans. Present study aimed to assess molecular diversity using candidate gene-based and genomic SSR markers because of their relevance to grain iron and zinc traits and to establish genetic relationships among the evaluated genotypes through cluster analysis.

MATERIALS AND METHODS

Research material comprised of fifty rice genotypes which included advanced Basmati breeding lines (F_6/F_7 generations), Basmati plus scented and non-Basmati varieties (Table 1). Leaf samples were collected from each fifty lines in aluminium foil and then samples were stored at -20 ^{0}C in the laboratory refrigerator for further use. Cetyl tri- methyl ammonium bromide (CTAB) DNA extraction method given by Doyle and Doyle (1990) was followed to isolate DNA from each leaf sample with some extent of variation as per the demand and accessibility of the resources in the experiment. Spectrophotometer was used to determine the purity and concentration of DNA. PCR amplification of DNA was performed in 0.2 mL Eppendorf tubes using a thermocycler. Final volume of 13.8 µL was maintained for each reaction mixture which comprised of template DNA, Taq DNA polymerase, PCR buffer with $MgCl_2$ & forward and reverse primer, and double-distilled water to make up the volume. For genotyping, 13 SSR markers, including both candidate gene-based and genomic SSR markers were used (Table 2). In PCR thermocycler, reaction proceed with denaturation temperature at 94◦C accompanied by marker specific annealing temperature and DNA extension at 72 ◦C which is performed by Taq DNA polymerase. Separation of amplified product obtained on 3% agarose gel employing horizontal gel electrophoresis. Visualization of DNA band and documentation was performed with the help of Alpha Imager gel documentation system (Agilent Technologies, USA). Marker scoring was done using binary units 0 and 1 score for absence and presence of bands among the genotypes. For data analysis, data matrix comprising of '1' and '0' was prepared depending upon the character. PIC value was calculated for each marker from binary data. Pairwise genetic similarity among genotypes was estimated using Jaccard's similarity coefficient, and cluster analysis was performed using the unweighted pair-group method with arithmetic mean (UPGMA) in NTSYS-pc version 2.2.

RESULTS

Assessment of the genetic diversity in rice is key to advancing rice grain quality enhancement techniques. Molecular markers have proven to be highly robust in characterizing genetic diversity in rice, as illustrated by many studies (Pradhan *et al*., 2020; Bollinedi *et al*., 2020). In the current study, fifty rice genotypes undergone characterized to assess genetic diversity using a total of thirteen SSR markers in relation to the grain iron and zinc content traits (Fig.1-3). These markers included four candidate gene-based markers (OsNRAMP7, OsVIT1, OsZIP1a and OsZIP3b) targeting iron and zinc, along with nine genomic SSR markers (RM44, RM17, RM3331, RM152, RM224, RM263, RM217, RM276, RM492) previously documented by various researchers for their association with grain iron and zinc. The SSR markers employed in the present study revealed substantial genetic diversity among the genotypes, with the majority exhibiting high Polymorphism Information Content (PIC) values, whereas RM492 and OsVIT1 showed a relatively low PIC value of 0.06 and 0.15. The PIC values ranged from 0.06 to 0.86, with the highest values observed for the marker RM152 followed by OsNRAMP7 (Table 3). Among the polymorphic markers, two generated one amplified fragment each, four generated two amplified fragment each, four produced three amplified fragment each and three produced 4 amplified fragment (Table 3). The number of amplified fragments per locus ranged from one (OsVIT1 and RM492) to 4 amplified fragments (RM217, RM276 and RM3331) with an average of 2.61 amplified fragments across the 13 markers. In the present study, variation in PIC value for both candidate gene- based markers and genomic SSR markers exhibited

differences in markers informativeness indicating considerable polymorphism within the germplasm studied, reflecting the overall genetic diversity within the studied population.
Genotypic data obtained after scoring the banding pattern of thirteen SSR score was used to prepare UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram which was based on Jaccard's similarity coefficient. Jaccard's similarity coefficient ranged from 0.56 to 0.96 (Fig. 4). UPGMA classified fifty genotypes of rice into 2 major clusters (A and B) which were further divided into various sub clusters. A and B are clustered at similarity coefficient of 0.56. Cluster A consisted of 41 genotypes. Most Basmati varieties, advanced Basmati breeding lines and scented varieties were clustered into major group A. Cluster B comprised of 9 genotypes. All the non-Basmati varieties such as RP 2068, MO1, PTB 33, Suraksha and TN1 were grouped into cluster B with exception of few advanced Basmati breeding lines (UPR 4218-15-1-1-1, UPR 4257-8-1-1-1, UPR 4257-8-1-1-3 and UPR 4259-2-1-1-2) present in it. The genotype UPR 4257-8-1-1- 1 was classified as a distinct subgroup B_2 in the dendrogram. The detailed relationships among individual genotypes are presented in Fig.4.

DISCUSSION

The genetic diversity in rice genotypes using SSR markers, and its relationship with micronutrient density has been investigated by many researchers (Pradhan *et al*., 2020; Raza *et al*., 2019; Singh & Singh, 2019). Notable benefits offered by SSR markers makes them valuable tools in plant breeding. Present study highlights the considerable diversity among rice genotypes and the evidence of this is marked by highly variable PIC values and high number of amplified fragments per locus. The number of amplified fragments and the reported polymorphism information content values varied indicating that the markers were different in their capacity to discriminate among the evaluated genotypes. This variation is useful for molecular characterization, because informative markers provide better resolution to assess genetic relationships in breeding material. The value obtained for number of amplified fragments per locus in present study are in close agreement with the value obtained by Vanitha *et al*., 2018; Matin *et al*., 2012. Markers exhibiting high PIC values suggested that most of those used in this study were moderately to highly informative, serving as reliable tools for genetic diversity.
The candidate gene-based markers exhibited considerable variations. Present result also corroborated by the genomic SSR markers which also produced similar result patterns. The candidate gene-based markers were OsZIP1a, OsZIP3b, OsNRAMP7 and OsVIT1. Several of these markers correspond to genes previously associated with processes involved in mineral transport and accumulation. Polymorphism at these loci in the present study showed molecular diversity among the rice genotypes studied but marker polymorphism did not define differences in grain Fe or Zn concentration. Direct phenotypic measurement of grain Fe or Zn content would be required therefore to determine whether the molecular variation detected at these loci was associated with grain Fe or Zn relevant phenotypic variation in the current germplasm. Li *et al*. (2023) confirmed the pivotal role of OsNRAMP7 in iron (Fe) accumulation in rice grains, underscoring its potential for biofortification. Consistently in our study, OsNRAMP7 indicated substantial genetic diversity within the germplasm population, but for establishing its association with Fe and Zn content in our genotypes need phenotypic characterization.
High PIC value indicated by genomic SSR markers were informative for differentiating the evaluated genotypes. But high PIC value alone without phenotypic data cannot establish association with grain Fe or Zn concentration. Several genomic SSR markers utilized in the present study have been previously reported by researchers to be associated with iron (Fe) and zinc (Zn) content in rice. Kumar *et al*. (2014) identified an association of the marker RM263 with a QTL for Fe content, while Garcia-Oliveira *et al*. (2009) showed that RM152, mapped on chromosome 8, was linked to a QTL for Zn content. Similarly, Chandu *et al*. (2020) reported the polymorphic nature of RM263 and its linkage with grain Fe and Zn traits. Furthermore, Raza *et al*. (2020) established the association of RM152 with Zn content, reinforcing its importance in grain Fe and Zn accumulation. Additionally, in our study, RM152 exhibited a high level of marker informativeness in the genotypes used in current study, indicating substantial genetic diversity. As several studies has reported its association with genomic region for Zn, it may be considered for further studies. Present findings require further validation of these marker with phenotypic characterization for grain iron and zinc.

CONCLUSION

Our study revealed molecular diversity among fifty rice genotypes using thirteen SSR markers, which included candidate gene-based and genomic SSR markers relevant to Fe- and Zn-related studies. Variation in the number of amplified fragments, marker informativeness and genetic similarity stipulated that considerable molecular diversity present in the evaluated germplasm. The UPGMA analysis classified the genotypes into two major class and showed both similarity and divergence among Basmati, scented, advanced breeding and non-Basmati material. Molecular data generated in the present study may help in further germplasm evaluation for future breeding programs. However, before specific genotypes or marker alleles can be recommended for micronutrient biofortification through marker-assisted selection, direct quantification of grain Fe and Zn concentrations and subsequent marker-trait validations are needed.

FUNDING

This study was completed without financial support from external organizations.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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Table 1: Rice genotypes used for SSR-based molecular characterization

S. No.	Genotypes	S. No.	Genotypes
1.	Taraori Basmati	26.	UPR 4259-2-1-1-3
2.	Pant Basmati 1	27.	UPR 4259-9-1-1-1
3.	Pant Basmati 2	28.	UPR 4259-9-1-1-2
4.	UPR 4217-15-1-1-1	29.	Pusa Basmati 1
5.	UPR 4217-15-1-1-2	30.	Improved Pusa Basmati 1
6.	UPR 4217-15-1-1-3	31.	Type 3
7.	UPR 4218-15-1-1-1	32.	Basmati 386
8.	UPR 4218-15-1-1-2	33.	Pusa Sugandh 5
9.	UPR 4218-15-1-1-3	34.	Pusa Sugandh 3
10.	UPR 4221-2-1-1-1	35.	Pant Sugandh Dhan 15
11.	UPR 4222-7-1-1-1	36.	Pant Sugandh Dhan 17
12.	UPR 4222-7-1-1-2	37.	Pant Sugandh Dhan 21
13.	UPR 4222-7-1-1-3	38.	Pant Sugandh Dhan 25
14.	UPR 4228-6-1-1-1	39.	Haryana Basmati 1
15.	UPR 4246-9-1-1-1	40.	Punjab Basmati 1
16.	UPR 4246-9-1-1-2	41.	Punjab Basmati 2
17.	UPR 4246-9-1-1-3	42.	Pusa Sugandh 2
18.	UPR 4250-8-1-1-1	43.	Basmati 385
19.	UPR 4250-8-1-1-2	44.	Basmati 370
20.	UPR 4250-8-1-1-3	45.	Pusa Basmati 1121
21.	UPR 4257-8-1-1-1	46.	RP 2068
22.	UPR 4257-8-1-1-2	47.	MO 1
23.	UPR 4257-8-1-1-3	48.	PTB 33
24.	UPR 4259-2-1-1-1	49.	Suraksha
25.	UPR 4259-2-1-1-2	50.	Taichung Native 1 (TN 1)

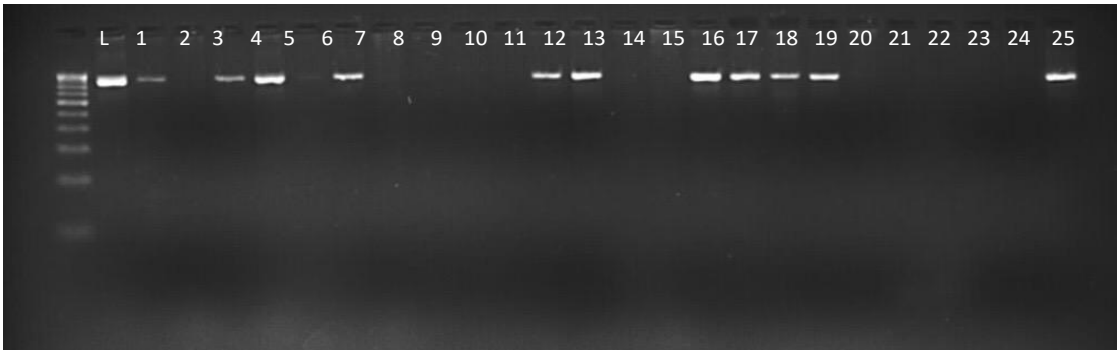
Table 2: SSR markers used for molecular characterization of the rice genotypes

S. No.	Primers	Chromosome	Annealing Temp.	Forward Sequence	Reverse Sequence
1.	**RM44**	8	54.8	ACGGGCAATCCGAACAACC	TCGGGAAAACCTACCCTACC
2.	**Os NRAMP 7**	12	60.0	CGGGGCAGACTAGTACCATAACG	CAGCAAGAGATAGCCATTGATCG
3.	**OsVIT 1**	-	54.8	AAGAGCGAGGCAGACCATTA	GGAATGGACGGTTTCCAGTA
4.	**OsZIP 1a**	3	59.0	GCTCTTGCTCGCTGCAATTC	CCAACCAGTACCCGTTCTCC
5.	**RM 17**	12	54.8	TGCCCTGTTATTTTCTTCTCTC	GGTGATCCTTTCCCATTTCA`
6.	**OsZIP 3b**	4	61.5	CCTGCTGAGGCTGAGTTGAA	CGAGAACAAAGTAACAGGCTGC
7.	**RM3331**	12	54.8	CCTCCTCCATGAGCTAATGC	AGGAGGAGCGGATTTCTCTC
8.	**RM152**	8	56.0	GAAACCACCACACCTCACCG	CCGTAGACCTTCTTGAAGTAG
9.	**RM224**	11	54.8	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG
10.	**RM263**	2	55.0	CCCAGGCTAGCTCATGAACC	GCTACGTTTGAGCTACCACG
11.	**RM217**	6	54.8	ATCGCAGCAATGCCTCGT	GGGTGTGAACAAAGACAC

12.	**RM276**	6	54.8	CTCAACGTTGACACCTCGTG	TCCTCCATCGAGCAGTATCA
13.	**RM492**	4	55.0	CCAAAAATAGCGCGAGAGAG	AAGACGTACATGGGTCAGGC

Table 3: DNA amplification pattern of SSR markers (number of amplified fragments, range of amplified band size in bp and PIC value) in rice genotypes

S. No.	Markers	No. of amplified fragments	No. of polymorphic bands	Range of amplified band size (bp)	PIC value
1	RM44	2	2	150-210	0.37
2	Os NRAMP 7	2	2	850-900	0.85
3	OsVIT1	1	1	900	0.15
4	OsZIP1a	2	2	850-900	0.46
5	RM 17	3	3	150-190	0.71
6	OsZIP3b	3	3	300-420	0.58
7	RM3331	4	4	100-180	0.64
8	RM152	3	3	180-200	0.86
9	RM224	2	2	200-210	0.59
10	RM263	3	3	190-220	0.61
11	RM217	4	4	120-180	0.69
12	RM276	4	4	90-170	0.58
13	RM492	1	1	230	0.06



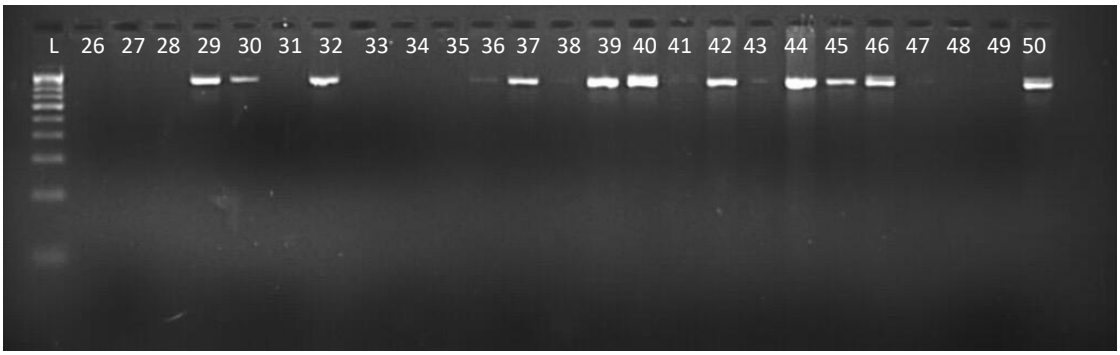
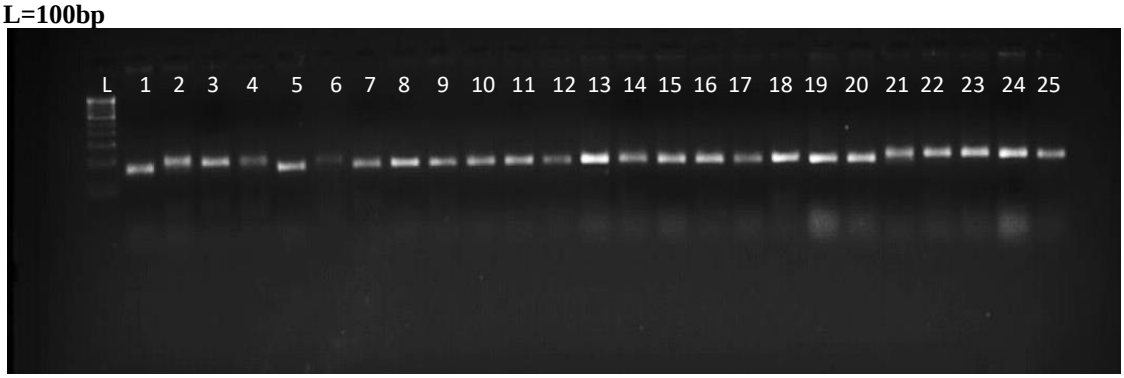


Fig. 1: Genotyping of 50 rice genotypes using OsNRAMP7
L=100bp



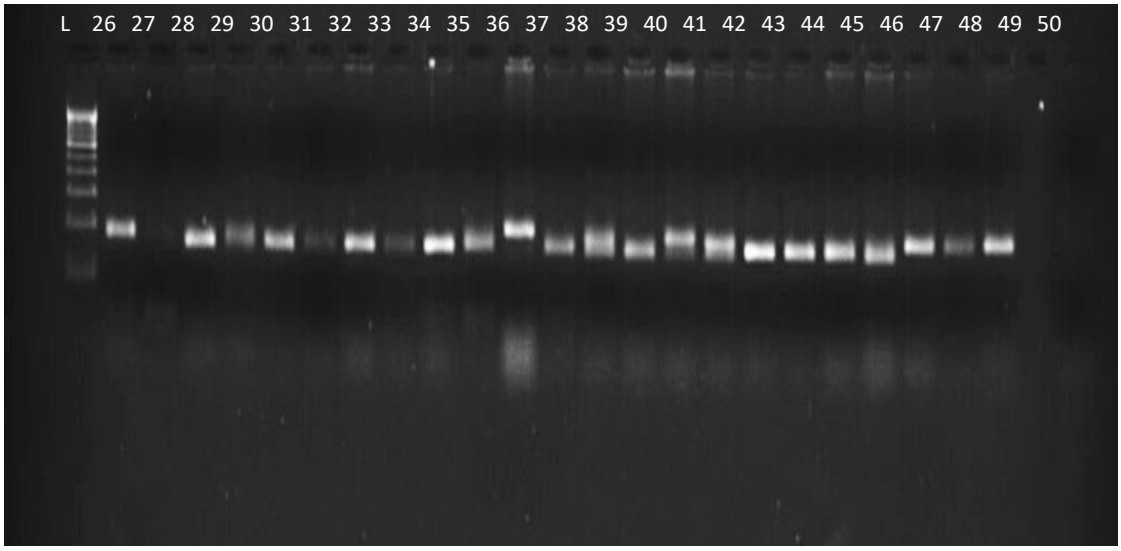
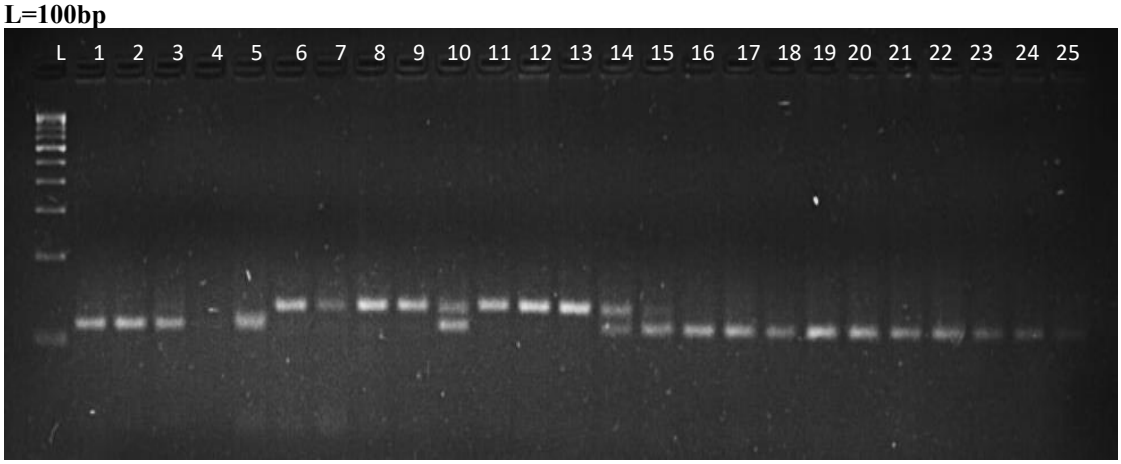


Fig. 2: Genotyping of 50 rice genotypes using marker RM263
L=100bp



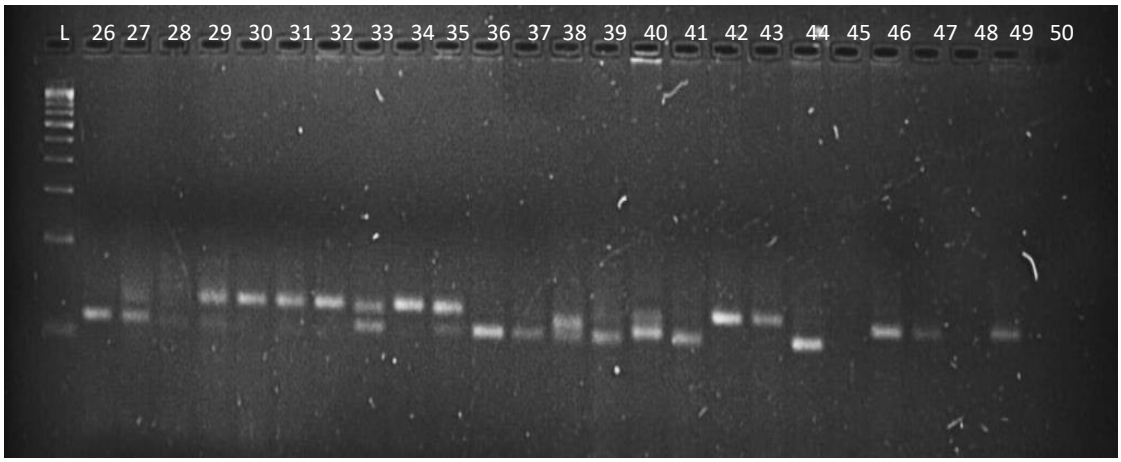


Fig. 3: Genotyping of 50 rice genotypes using marker RM3331
L=100bp

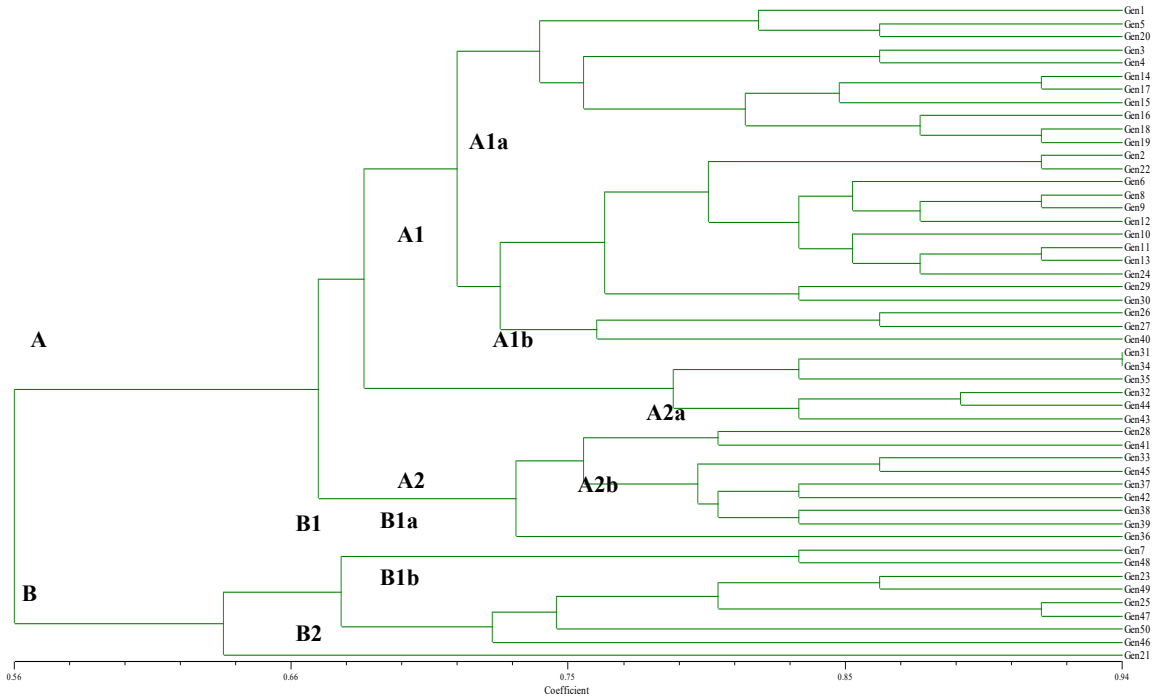


Fig.4: UPGMA dendrogram showing molecular relationships among 50 rice genotypes based on Jaccard's similarity coefficients derived from 13 SSR markers

Gen 1= Taraori Basmati, Gen 2= Pant Basmati 1, Gen 3 = Pant Basmati 2, Gen 4= UPR 4217-15-1-1-1, Gen 5= UPR 4217-15-1-1-2, Gen 6 = UPR 4217-15-1-1-3 Gen 7= UPR 4218-15-1-1-1, Gen 8 =UPR 4218-15-1-1-2, Gen 9 = UPR 4218-15-1-1-3, Gen 10 = UPR 4221-2-1-1-1, Gen 11 = UPR 4222-7-1-1-1, Gen 12 = UPR 4222-7-1-1-2, Gen 13 = UPR 4222-7-1-1-3, Gen 14 = UPR 4228-6-1-1-1, Gen 15= UPR 4246-9-1-1-1, Gen 16= UPR 4246-9-1-1-2, Gen 17= UPR 4246-9-1-1-3, Gen 18 = UPR 4250-8-1-1-1, Gen 19 = UPR 4250-8-1-1-2, Gen 20 = UPR 4250-8-1-1-3, Gen 21 = UPR 4257-8-1-1-1, Gen 22 = UPR 4257-8-1-1-2, Gen 23 = UPR 4257-8-1-1-3, Gen 24 = UPR 4259-2-1-1-1, Gen 25 = UPR 4259-2-1-1-2, Gen 26 = UPR 4259-2-1-1-3, Gen 27 = UPR 4259-9-1-1-1, Gen 28 = UPR 4259-9-1-1-2, Gen 29 = Pusa Basmati 1, Gen 30=Improved Pusa Basmati 1, Gen 31 = Type 3, Gen 32 = Basmati 386, Gen 33 = Pusa Sugandh 5, Gen 34= Pusa Sugandh 3, Gen 35 = Pant Sugandh Dhan 15, Gen 36 = Pant Sugandh Dhan 17, Gen 37 = Pant Sugandh Dhan 21, Gen 38 = Pant Sugandh Dhan 25 , Gen 39 = Haryana Basmati 1, Gen 40 = Punjab Basmati 1 , Gen 41 = Punjab Basmati 2, Gen 42 = Pusa Sugandh 2, Gen 43 = Basmati-385 , Gen 44= Basmati 370, Gen 45 = Pusa Basmati 1121, Gen 46 = RP 2068, Gen 47 =MO 1, Gen 48 =PTB 33 , Gen 49 =Suraksha and Gen 50 = TN 1

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