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Research Article

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Molecular Characterization of Rice (*Oryza sativa* L.) Genotypes by using DNA Markers

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ABSTRACT

Rice (Oryza sativa L.) is a "Global Grain" cultivated widely across the world and feeds millions of mankind, is the staple food for more than half of the human population, and in Asia alone more than two billion people depend on rice and its products for their food intake. Rice has become deeply embedded in the cultural heritage of Asian societies and is the life, heart and soul of the people throughout Asia and can be considered as "Rice Basket" of the world, as 90 per cent of world's rice is grown and consumed by 60 per cent of population and where, about two-thirds of world's poor live (Khush and Virk, 2005). For molecular characterization, ten rice genotypes by using seven SSR primers among these only five primers viz., RM193, RM190, RM186, RM234 and RM223 showed polymorphism in all genotypes, PIC value ranges from 0.36 to 0.39 and major allele frequency ranged from 0.42 (RM 186) to 0.50 (RM 190) with an average of 0.46 per marker. The genetic dissimilarity estimates for ten genotypes were employed to generate dendrogram by using tree construction with NTSYS Software. Cluster I consists of four genotypes (Gangavathi emergency, IET-25546, DRR-Dhan-44 and RP-Bio-226), Cluster II consists of three genotypes (IET-23304, IET-22066 and IABT-17), Cluster III consists of two genotypes (IET-255051 and IET-26282) and Cluster IV consists of one genotype (GNV-11-09). Molecular characterization of the genotypes gives precise information about the extent of genetic diversity which helps in the development of an appropriate breeding programme.

Key words: *Rice, Global Grain, Khush and Virk*

INTRODUCTION

The cultivated rice plant (*Oryza sativa* L.) belongs to the family Oryzae. It is distributed throughout the tropics and subtropics, however it is grown more easily in the tropics. Rice is the staple food for more than half of the world's population and it is nutritionally rich in 90 per cent carbohydrate, 8 per cent protein and 2 per cent fat. Molecular characterization of the genotypes gives precise information about the extent of genetic diversity which helps in the development of an appropriate breeding programme.

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The most widely adopted markers used for genetic divergence studies among various genotypes are Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR), Inter specific Simple Sequence Repeats (ISSR) and Restriction Fragment Length Polymorphism (RFLP), having their own advantages and disadvantages and needs careful evaluation before effectively developed for diversity analysis[10]. DNA fingerprinting techniques like RFLP, RAPD, SSR, ISSR and AFLP etc. have been of significant use in discriminating varieties[9,5].

MATERIAL AND METHODS

The genotypes used for molecular characterization were Gangavathi emergency, DRR Dhan, RP-Bio-226, IABT-17, GNV-11-09, IET-22066, IET-25546, IET-26282, IET-255051 and IET-23304 and rice microsatellite markers viz., RM 193, RM 190, RM 186, RM 234 and RM 223

DNA Extraction

Twenty five days old seedlings were used for DNA extraction. DNA was prepared as per the modified Cetyltrimethyl ammonium bromide (CTAB) method[1].

1. Pre-chilled the mortar-pestle at -80 °C for 15 minutes prior to the start of the experiment; alternatively, it can be pre-chilled at 20 °C for one hour.
2. Two gram of fresh leaf was taken and ground with liquid nitrogen to make fine powder.
3. Added free warmed (65 °C) extraction buffer (10 µl) in 50 µl centrifuge tube.
4. Kept in water bath at 65 °C for 10-15 minutes with intermittent mixing.
5. Samples were cooled to room temperature.
6. Equal volume of chloroform: isoamylalcohol (24:1) were added and mixed by inverting.
7. Spinned at 10000 rpm for 10 minutes.
8. Taken supernatant to fresh tube carefully.
9. Equal volume of prechilled isopropanol added and mixed gently.
10. Allowed for 1-2 hours at -20 °C or overnight.
11. DNA was spooled or spun down at 10000 rpm for 10 minutes.
12. DNA pellet re-suspended in TE buffer.

Quantification of DNA- Agarose gel electrophoresis

2 µl of each sample was loaded on 0.8 per cent agarose gel and was electrophoresed at 100 volts. After electrophoresis gel was stained with ethidium bromide solution (10 µg/ml) for 15 min and viewed under trans-illuminator (Syngene UVI Tec DOC- 008XD). Quantification was done based on the intensity of the bands and also quantified by using nano photometer equipment.

Normalization of DNA concentration for PCR

Normalization of DNA was done to bring all DNA concentrations to a relatively equal level (20 ng /µl) by appropriate dilutions. Dilutions were done with TE buffer.

Microsatellite marker analysis

Informative Microsatellite primer pairs were used for genotyping of the paddy genotypes. These were selected based on previous published authors of Mahender *et al*.[7].

Table 1. List of primers used for varietal characterization of rice genotypes

Sl no.	Markers	Forward primers (5'-3')	Revere primers (3'-5')
1	RM193	CGCCTCTTCTTCCTCGCCTCCG	CGGGTCCATCCCCCCTCTCCTC
2	RM 190	CTTTGTCTATCTCAAGACAC	TTGCAGATGTTCTTCCTGATG
3	RM186	TCCTCCATCTCCTCCGCTCCCG	GGGCGTGGTGGCCTTCTTCGTC
4	RM234	ACAGTATCCAAGGCCCTGG	CACGTGAGACAAAGACGGAG
5	RM 223	GAGTGAGCTTGGGCTGAAAC	GAAGGCAAGTCTTGGCACTG

Amplification reaction mixture for SSR markers

Amplification reaction mixture for SSR markers was prepared in 1.2 ml thin walled PCR tubes containing following components in a total volume of 15µl for both specific marker trait analysis and varietal characterization of rice genotypes.

Sl. No.	Components	Quantity (µl/reaction)
1	10X Assay buffer with 25mM $Mgcl_2$	2
2	dNTPs mix (2.5 mM each)	1
3	Primer (10 pm/µl)	1.2
4	Template (15 ng/µl)	1
5	Sterile distilled water (µl)	9
6	Taq DNA polymerase (5 U/µl)	0.30

Except template, the master mix was distributed to PCR tubes (15 µl/tube) and later 1.5 µl of template DNA from the respective genotypes was added making the final volume of 15 µl.

The thermo profile PCR for SSR

The PCR amplification for SSR analysis for both specific marker trait analysis and varietal characterization of rice genotypes was performed according to Williams *et al.*[13], with certain modifications. The amplification conditions were as follows,

Sl no.	Steps	Temperature (^{o}C)	Duration (min.)	Number of cycles
1	Initial denaturation	94	5.00	1
2	Denaturation	94	1.00	
3	Annealing	36	1.00	40
4	Extension	72	1.00	
5	Final extension	72	0.00	1
6	Dump	4	-	-

After the completion of the PCR, the products were stored at 4 ^{o}C until the gel electrophoresis was done.

Separation of amplified products by Agarose Gel

The amplified products from each tube along with 2µl of loading dye (bromophenol blue) were separated on 1.4 per cent agarose gel containing ethidium bromide at 70 volts (<5 volts per cm of gel) using 1X TAE buffer of pH 8.0. Lambda DNA double digest was used as DNA molecular weight marker. The gel was photographed by using documentation system (Bio Rad) in case of both specific marker trait analysis and varietal identification of rice genotypes.

Gel electrophoresis

Agarose gel of (3 per cent) was prepared using electrophoresis grade agarose in a volume of electrophoresis buffer (1x TAE) sufficient for constructing a gel (300 ml for 18 x 30 cm gel). Ethidium bromide was added at concentration of 0.5 µg/ml of gel. The gel was allowed to set fully before removing the comb

and loading the sample. 5 µl of loading dye was added to 20 µl of PCR products and mixed well before loading into the wells. Care was taken to prevent mixing of samples between the wells. A voltage of 1-5 v/cm was given for a time period of three hours for separation of PCR fragments. After electrophoresis, the DNA banding pattern was viewed under UV light and documented; this was followed in both specific marker analysis and varietal characterization of rice genotypes.

Scoring of bands

The bands generated by SSR primers were scored based on presence (1) and absence (0) and location of bands in both specific marker analysis and varietal characterization.

Statistical analysis

The statistical analysis of the data on individual character was carried out on the mean values of each genotype using WINDOSTAT package, 8.1 version. The characters were subjected to statistical analysis as per Federer in order to assess the variability among the genotypes. Critical differences were calculated at 1 and 5 per cent level wherever 'F' test was significant in case of both specific marker trait analysis and varietal characterization.

Grouping of genotypes

Pooled data on characterization of 10 genotypes were grouped as per the high vigour and low vigour banding pattern.

RESULTS AND DISCUSSION

A total of seven SSR markers were used to screen the ten genotypes, out of which five markers produced amplification and showed polymorphic bands and other two markers showed monomorphism. Ten rice genotypes were characterized by using SSR primers. The PIC value, no. of alleles and MI value of five SSR primers are presented in Table 1.

Molecular markers have been used in genetic improvement programmes to study genetic diversity and to select parents for planning crossing between parents from divergent backgrounds and to know marker trait association[4]. The present study assessed the genetic base of ten rice genotypes employing ten microsatellite simple sequence repeats (SSR) to assess the molecular diversity and characterization of rice genotypes. SSR had much more polymorphism than most of other DNA markers, and is co-dominant and larger in quantity. Therefore, the high polymorphic information content of SSR had prompted the application of microsatellites as molecular markers in fingerprinting.

The SSR markers used in the present study were scorable and unambiguous. Out of seven markers used only five were polymorphic allele and remaining two markers were monomorphic in nature. The number of alleles detected per marker were 2 and 3 with an average of 2.40. The alleles showed high degree of polymorphism, with cent per cent polymorphic bands in five SSR markers. It suggests that the genotypes used in present study shown as genetic divergence.

RM 193 primer produced 2 alleles with PIC value 0.48 and MI value 0.96. All the ten genotypes amplified at different base pairs (bp). Among the ten genotypes, four genotypes (IABT-17, GNV-11-09, IET-255051 and IET-25546) amplified at 120 bp, three genotypes (Gangavathi emergency, IET-23304 and IET-26234) amplified at 130 bp, two genotypes (RP-Bio-226 and DRR-Dhan-44) amplified at 140bp and only one genotype (IET-26282) amplified at 110bp.

RM 190 marker produced 2 alleles with PIC value and MI value 0.50 and 1.00 respectively. All the genotypes amplified at different bp. The four genotypes (Gangavathi emergency, RP-Bio-226, IET-26282 and IET-23304) amplified at 200 amplicons, four genotypes (IET-26234, IET-255051, IABT-17 and DRR-Dhan-44) amplified at 180 amplicons, and two genotypes (IET-25546 and GNV-11-09) at 150bp.

Three alleles were produced by marker RM 186 and produced PIC value 0.42 and MI value 1.26. The four genotypes (Gangavathi emergency, IABT-17, RP-Bio-44 and DRR-Dhan-44) showed amplification at 160 base pairs, three genotypes (IET-255051, IET-25546 and IET-23304) amplified at 170 bp, two genotypes (IET-220551 and IET-2234)

amplified at 150 bp and one genotype (IET-26282) amplified at 140bp.

All the genotypes *viz.*, Gangavathi emergency, RP-Bio-226, DRR-Dhan-44, IABT-17, GNV-11-09, IET-23304, IET-22066, IET-255051, IET-26282 and IET-25546 amplified at different base pairs for marker RM 234. The marker RM 234 brought out 3 alleles with the PIC value and MI value 0.46 and 1.38 respectively. Out of 10 genotypes, five genotypes (IET-25546, RP-Bio-226, DRR-Dhan-44, GNV-11-09 and IET-23304) showed amplification at 130 base pairs and five genotypes (IET-22066, Gangavathi emergency, IET-26282, IET-255051, IET-26234, IABT-17and IET-25546) amplified at 120 bp.

Amplification of all the ten genotypes was found to be at different base pairs with the marker 223. The four genotypes (Gangavathi emergency, IET-26282, GNV-11-09, and IET-255051) amplified at 120 bp, remaining four genotypes (DRR-Dhan-44, IET-25546, IET-23304 and RP-Bio-226) amplified at 130 bp and the two genotypes amplified at 110bp.

The five SSR markers RM 193, RM 190, RM 186, RM 234 and RM 223 showing polymorphism, these polymorphic markers amplified a total of 13 alleles with an average of 2.60 alleles per markers in parental lines surveyed. Out of five markers, two markers amplified 2 allele (RM 193 and RM 190), other three markers (RM 186, RM 234 and RM 223) amplified 3 alleles. These alleles average was found to be 2.60. The PIC (Polymorphism Information Content) value ranged from 0.42bp (RM 186) to 0.50 (RM 190) with an average of 0.46 per marker and MI (Marker Index) value ranged from 0.96 (RM 193) to 1.38 (RM 234) with an average of 1.18.

The genetic dissimilarity estimates for ten genotypes were employed to generate dendrogram by using tree construction with NTYSS Software (Fig. 1). Ten genotypes were distributed in four main clusters. Cluster I consists of 4 genotypes *viz.*, Gangavathi emergency, IET-25546, DRR-Dhan-44, RP-Bio-226), cluster II possessed 3 genotypes (IET-23304, IET-22066 and IABT-17), cluster III consists of 2 genotypes (IET-255051 and IET-26282) and cluster IV possessed only one genotype i.e., GNV-11-09 (Table 2).

Genetic diversity is a pre requisite for selection of various plant breeding programmes and the diversity at genomic level can be detected by several means. Though a range of plant morphological traits are currently available and are used for distinguishing genotypes, environment plays an important role in influencing their expression. Molecular level investigations reveal the real differences among the genotypes. Molecular markers have been used in genetic improvement programmes to study genetic diversity and to select parents for planning crossing between parents from divergent backgrounds and to know marker trait association[4]. The present study assessed the genetic base of ten rice genotypes employing ten microsatellite simple sequence repeats (SSR) to assess the molecular diversity and characterization of rice genotypes. SSR had much more polymorphism than most of other DNA markers, and is co-dominant and larger in quantity. Therefore, the high polymorphic information content of SSR had prompted the application of microsatellites as molecular markers in fingerprinting.

The SSR markers used in the present study were scorable and unambiguous. Out of seven markers used only five were polymorphic allele and remaining two markers were monomorphic in nature. The number of alleles detected per marker were 2 and 3 with an average of 2.40. The alleles showed high degree of polymorphism, with cent per cent polymorphic bands in five SSR markers. It suggests that the genotypes used in present study shown genetic divergence.

Polymorphism information content (PIC) value is reflection of allele diversity and frequency among the cultivars. PIC value of each marker can be evaluated on the basis of its alleles. PIC value varied significantly for all the studied SSR loci. In the present study, the level of polymorphism among the 10 genotypes was evaluated by calculating PIC

values for each of the SSR loci. The PIC values ranged from 0.42 (RM 186) to 0.50 (RM 190) with an average of 0.46. MI value ranged from 0.96 (RM 193) to 1.38 (RM 234) with an average of 1.18. Markers with PIC values of 0.5 are highly informative for genetic studies and are extremely useful in distinguishing the polymorphism rate of a marker at a specific locus. Presence of polymorphism between genotypes revealed that the presence of genetic diversity at molecular level. Similar studies were carried out by Gulhanercan *et al*.[3], varied levels of polymorphism have been reported by Multan and Lyon[8] in cotton, Encheva *et al*.[2], in sunflower, Silvancreste *et al*.[11], in groundnut and Subramanian *et al*.[12], in groundnut genotypes.

Table 2. DNA polymorphism in rice genotypes obtained using combination of SSR primers

Sl no.	Primers	Allele No.	PIC value	MI value
1	RM 193	2	0.48	0.96
2	RM 190	2	0.50	1.00
3	RM 186	3	0.42	1.26
4	RM 234	3	0.46	1.38
5	RM 223	3	0.44	1.32
	Mean	**2.60**	**0.46**	**1.18**

Where, PIC = Polymorphic Information Content, MI = Marker Index

Table 3. Distribution of ten rice genotypes in different clusters in rice genotypes

Sl no.	Cluster name	Number of genotypes	Genotypes
1	Cluster I	4	Gangavathi emergency, IET-25546, DRR-Dhan-44 and RP-Bio-226
2	Cluster II	3	IET-23304, IET-22066 and IABT-17
3	Cluster III	2	IET-255051 and IET-26282
4	Cluster IV	1	GNV-11-09

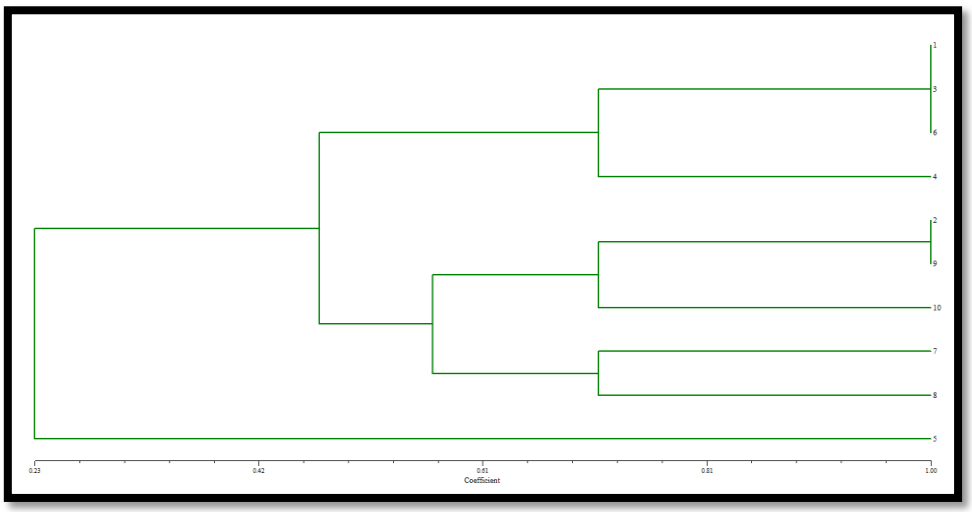

Fig 1. Dendogram representing distribution of ten rice genotypes in four different clusters

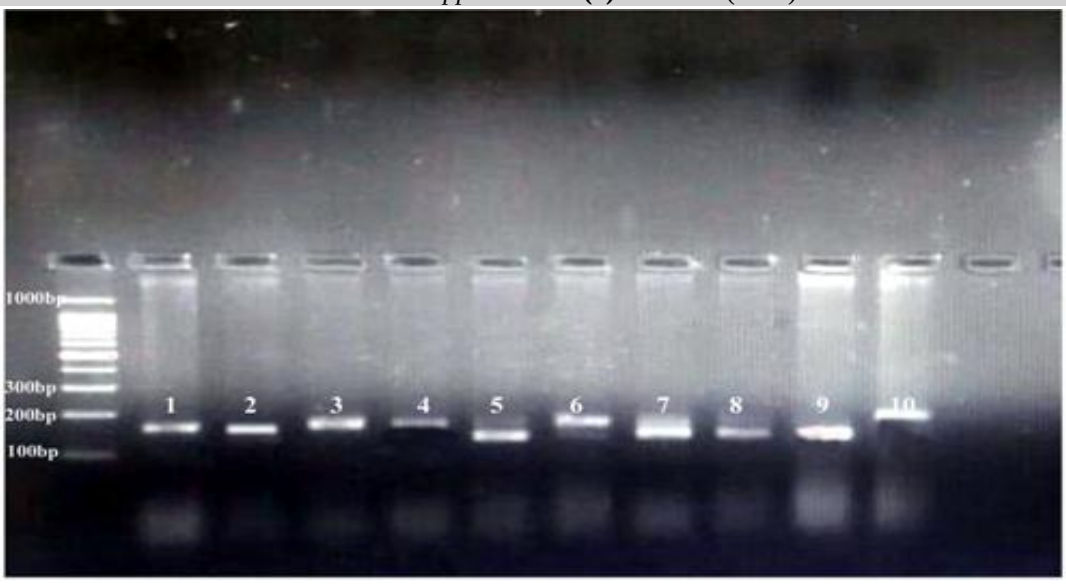

Plate 1. PCR amplification of rice genotypes with RM193 primer

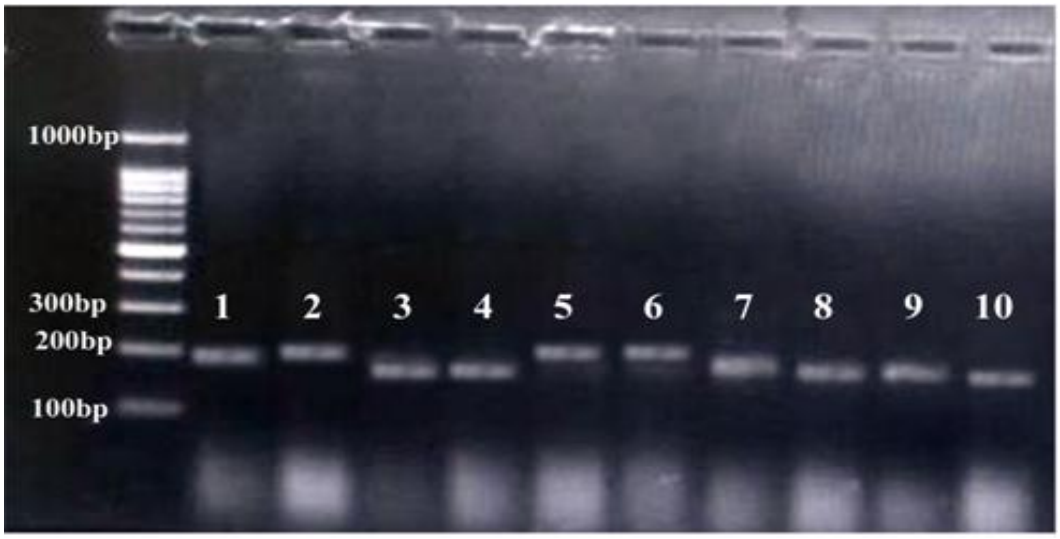

Plate 2. PCR amplification of rice genotypes with RM190 primer

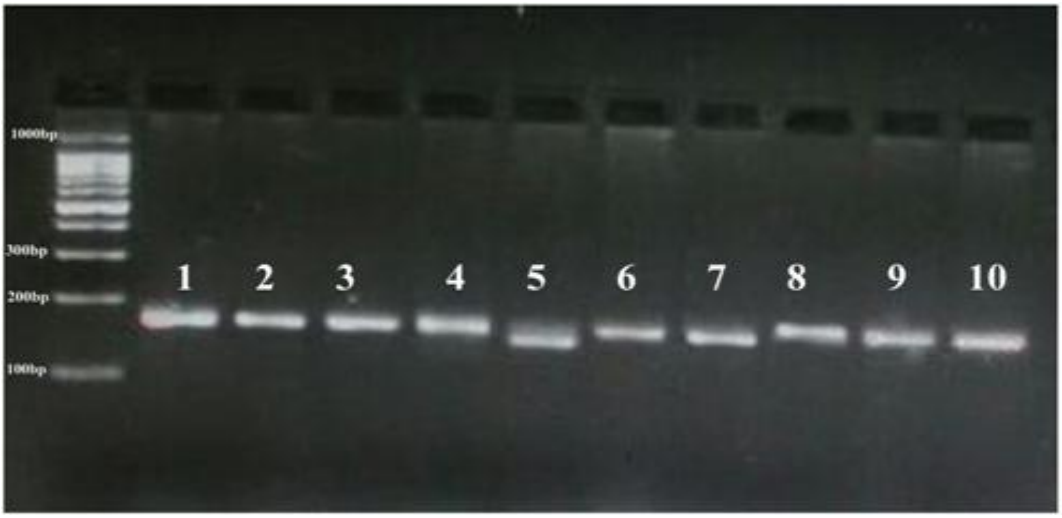

Plate 3. PCR amplification of rice genotypes with RM186 primer

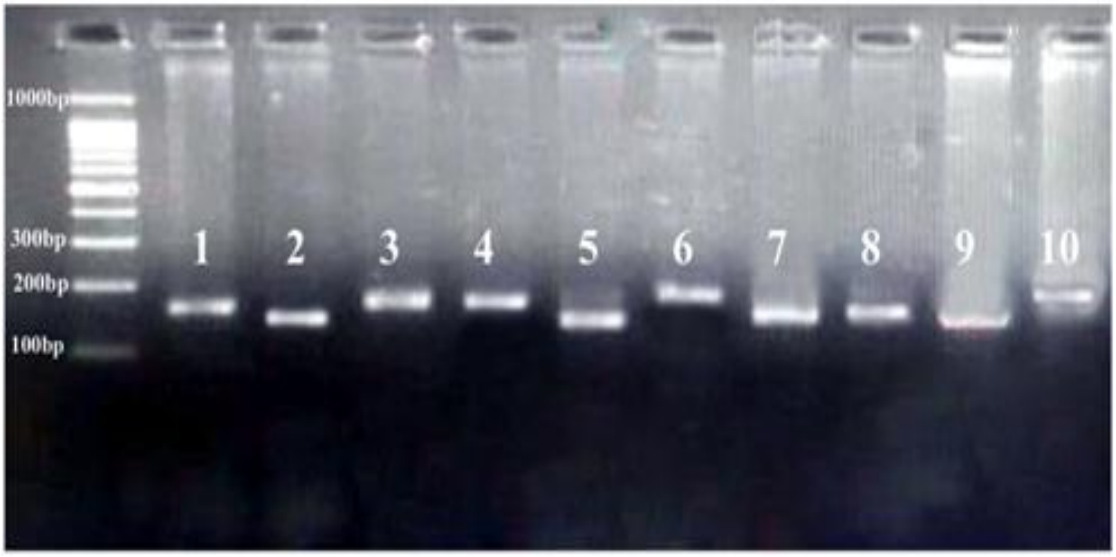

Plate 4. PCR amplification of rice genotypes with RM234 primer

Plate 5. PCR amplification of rice genotypes with RM223 primer

G1-Gangavati emergency G2- IET-23304 G3- GNV-11-09 G4- IET-25546
G5- RP-Bio-226 G6-IET-26282 G7- IABT-17 G8- IET-255051
G9- DRR-Dhan-44 G10- IET-26234

CONCLUSIONS

Molecular characteristic of important ten genotypes showed polymorphism for five SSR primer *viz.*, RM193, RM190, RM186, RM234 and RM223 which can be used for identifying the genotypes. Molecular characterisation using SSR primers showed polymorphism among different genotypes.

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