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Genetic diversity and molecular analysis for fertility restorer genes in Rice (*Oryza sativa* L.) for wild abortive (*WA*) cytoplasm using microsatellite markers

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Wild abortive types of male sterile lines are commercially used in the production of hybrid varieties of rice. The present study was carried out with the objective to assess genetic diversity among 22 rice genotypes representative of restorer, maintainer and male sterile lines using SSR markers. Nearly 30 SSR markers have been used, of which 9 markers were selected from the genomic regions of chromosome 1 and 10 on which *Rf3* and *Rf4* genes were located. Among 30 SSR markers used, 25 SSR loci generated polymorphic patterns and a total of 231 alleles were amplified. The number of alleles per locus ranged from 5 to 17 with a mean of 9.4 alleles per locus. The PIC values for 25 SSR markers varied from 0.74 (RM195, RM10318, and RM258) to 0.92 (RM302). The present study revealed that molecular characterization using SSR markers can be an efficient tool for genotyping the varieties with reasonable accuracy.

Key words: Cytoplasmic Male Sterility (CMS), Fertility restoration, Wild Abortive cytoplasm (*WA*), SSR markers

Introduction

Rice is the staple food for the millions of people in the world (Hashemi *et al*., 2009). Hybrid rice technology is considered as one of the promising, practical, sustainable and eco-friendly options to break the yield ceiling in rice (Sheeba *et al*., 2009). Production of rice hybrids using a CMS system is based on cytoplasmic male sterility and fertility restoration systems, hence it involves three lines of male sterile (A), maintainer (B) and Restorer (R). Cytoplasmic male sterility is caused by lesion or rearrangement of mitochondrial genome resulting in its inability to produce functional pollen. But CMS can be restored

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by nuclear genes governing fertility restoration (Nematzadeh and Kiani, 2010). Three primary types of CMS are known in rice i.e. wild-abortive (*WA*), Bao tai (BT) and Honglian (HL) whose inheritance patterns and physiological characteristics have been extensively investigated. *WA* type CMS belongs to sporophytic abortion, which fails to produce normal pollen and finally leads to formation of typical abortive pollen (Sattari *et al*., 2008).

CMS and restorer lines are difficult to identify by using conventional methods because the procedure for identification is labor intensive. Even though some morphological markers have been present linked to CMS governing genes, their unstable pattern of inheritance leads to erroneous conclusions. Though morphological markers have been employed in assessing the underlying genetic diversity of a species, the accuracy of these as sole markers is not always confirmative. The availability of low number of morphological and biochemical markers, their poor and unknown genetic control, environmental influence and phenotypic expression, stage specific identification and procedural difficulties are known impediments in using them alone as genetic markers for diversity analysis. Hence employment of molecular markers is the best strategy for discriminating and differentiating CMS line from restorer lines. Microsatellite or SSR markers are simple tandemly repeated, di-to tetra nucleotide sequence motifs flanked by unique sequences. They are valuable as genetic markers because they detect high levels of allelic diversity, are co-dominant, and are easily and economically assayed by PCR (Jain *et al*., 2004). Previously, SSR markers have been used for studying genetic diversity and fine mapping of fertility restoration genes by Ahmadikhah *et al*. (2006), Jing *et al.* (2001), Bazrkar *et al.* (2008), Sheeba *et al*. (2009), and Sattari *et al.* (2009). Therefore, the present study was conducted with the objective of characterization and documentation of released rice varieties of Gujarat, India for the presence of fertility restorer genes (*Rf*3 and *Rf*4) using SSR markers.

Materials and methods

Plant materials

In the present investigation 22 rice genotypes (Table 1.), comprising A (male sterile), B (maintainer) and R (restorer) lines were used to study genetic diversity and molecular analysis of fertility restorer genes by SSR markers. All the genotypes were collected from Main Rice Research Station (MRRS), Anand Agricultural University, Nawagam, Gujarat, India.

Table 1. List of genotypes used in the present study

Sr. No.	Genotypes	Pedigree
1	IR-24	IRRI cultivar (R)
2	GR-3	N-19 X IR-9-60
3	GR-5	Selection from local cultivar NVS-18
4	GR-6	GR-3 X Pusa-33 (M)
5	GR-7	GR-3 X Basmati. 370 (R)
6	GR-8	Selection from pure line Vyara-55,IET17510
7	GR-9	Sathi-34-36 X CR-544
8	GR-11	Zinia-31 X IR-8-246 (M)
9	GR-12	GR-4 x IR-64
10	GR-13	GR-11 x IET-14726
11	GR-101	IR-8 X Pankhali-203 (R)
12	GR-102	IR-8 X Pankhali-203
13	GR-103	GR-11 X Masuri
14	GR-104	GR-101 X Basmati.370(R)
15	AAUDR-1	Sathi34-36xDadri Kolam
16	Ashoka-200F	Kalinga-III x IR 64
17	Dandi	PNL2×IET-8320
18	Gurjari	Asha X Kranti
19	Narmada	TN-1 X Basmati.370
20	Pankhali-203	Selection from local cultivar Pankhali
21	Sathi 34-36	Selection from local cultivar Sathi
22	SK-20	Selection from local cultivar Sukhvel (M)

(R)= Restorer and (M)= Maintainer

DNA extraction and SSR analysis

DNA was extracted from fresh leaves by the Cetyl Trimethyl Ammonium Bromide method (Ahmadikhah *et al*., 2007) with some modifications and the quality was confirmed through Nanodrop N.D. 1000 software (ver 3.3.0). For SSR analysis a total of 30 SSR primer pairs (MWG: - Biotech AG, India) were used for PCR amplification which spread across different chromosomes of rice. Each polymerase chain reaction was carried out in 20 µl reaction volume containing 50 ng of DNA, 10X polymerase buffer (10x tris with 20 mM $MgCl_2$), 25 mM dNTPs, 0.5 µl of each primer (10Pmol), 1 unit of Taq polymerase (Fermentas), 0.5 µl of 5% formamide and 1 µl of 5mM spermidine using Eppendorf and Applied biosystem thermal cyclers. Thermal cycler programme for PCR comprised 95^0C for 5 minutes for initial denaturation, followed by 36 cycles of 95^0C for 45 seconds, 55 to 65^0C for 45 seconds, 72^0C for 45 seconds and ending up with 7 minutes at 72^0C for the final extension. The annealing temperature was adjusted based on the specific requirements of different primer combinations. The PCR products were resolved by

electrophoresis in 2.5% agarose gel containing 0.5 µg/ml of Ethidium Bromide prepared in 1X TBE buffer at a constant voltage of 80v for period of 2 hrs. The gel was visualized in UV transilluminator and documented using SYNGENE GENESNAP G-BOX gel documentation system.

Analysis for SSR markers

Allelic frequency, allele length, expected heterozygosity and standard deviation for the SSR markers were calculated using identity software (Wagner and Sefc, 1999). Clear and distinct bands amplified by SSR primers were scored for the presence (1) or absence (0) of the corresponding bands among the genotype. The data were entered in to binary matrix and subsequently analyzed using NTSYSpc ver 2.2 (Rohlf *et al*., 1994). Coefficient of similarity was calculated by using Jaccard's coefficient by SIMQUAL sub-function and cluster analysis was performed by agglomerative technique using the UPGMA (Un-weighted Pair Group Method with Arithmetic mean) method by SAHN clustering sub-function of NTSYSpc. Relationships among rice genotypes were graphically represented in the form of dendrogram.

Results

Analysis for Rf3 and Rf4 gene by SSR markers (Table:3)

In the present investigation, analysis for *Rf3* gene was performed by two SSR markers viz., RM315 and RM443 which were earlier reported to be linked to this gene (Bazrkar *et al*., 2008). These two markers amplified 18 alleles. The PIC values of these markers were 0.75 and 0.88, respectively. Analysis for *Rf4* gene was performed by 12 SSR markers RM171, RM258, RM1108, RM3530, RM5359, RM6100, RM6737, RM8146, RM6344, RM10305, RM10313 and RM10318. These markers amplified 108 alleles. The PIC values for these markers which were reported earlier as flanking markers for Rf4 gene (Hashemi *et al*., 2007) were 0.74 (RM258, RM10318) and 0.89 (RM10313), respectively.

Analysis for genetic diversity by SSR markers (Table: 2)

The analysis for genetic diversity was performed using 11 SSR markers. They are RM195, RM223, RM208, RM284, RM302, RM310, RM342A, RM535, RM576, RM7003 and RM7102. The number of alleles per locus ranged from 5 (RM195) to 17 (RM302). The PIC value for these markers varied from 0.74 to 0.92.

Table 2. Details of SSR markers used for genetic diversity

Sr. No.	Primers	No. of Alleles	Range of allele length (bp)	Allele frequency (%)	PIC values
1	RM195	5	298-325	4.5-36.3	0.74
2	RM208	5	268-289	11.7-39.4	0.77
3	RM223	8	143-166	5.8-29.4	0.82
4	RM284	8	134-160	4.5-22.7	0.89
5	RM302	17	121-205	2.2-13.6	0.92
6	RM310	15	97-137	4.7-14.2	0.91
7	RM342A	12	129-164	2.2-20	0.87
8	RM535	9	390-461	6.2-25	0.83
9	RM576	11	237-323	5.5-16.6	0.89
10	RM7003	8	112-126	4.5-27.2	0.83
11	RM7102	10	165-212	5.8-17.6	0.89

Table 3. Details of SSR markers used for fertility restoration

Sr. No.	Primers	No. of Alleles	Range of allele length(bp)	Allele frequency (%)	PIC values
1	RM171	9	314-363	4.5-18.1	0.86
2	RM258	6	122-156	4.7-38	0.74
3	RM1108	6	122-158	5-20	0.87
4	RM3530	9	193-180	2.2-22.7	0.84
5	RM5359	9	189-217	4.7-28.5	0.83
6	RM6100	9	161-189	5-40	0.78
7	RM6737	9	147-218	4.7-28.5	0.83
8	RM8146	13	107-237	2.2-18.1	0.84
9	RM6344	8	94-117	2.2-31.8	0.80
10	RM10305	12	155-187	4.5-18.1	0.88
11	RM10313	9	203-274	4.7-33.3	0.82
12	RM10318	6	165-184	5.8-41.1	0.74
13	RM315	6	126-140	4.5-36.3	0.75
14	RM443	12	154-246	2.5-15	0.88

Cluster analysis of molecular data

The data generated from 25 SSR markers for 22 rice genotypes were utilized for calculating genetic similarity coefficient and construction of dendrogram. The maximum similarity index of 0.3 was obtained between genotype GR101 and GR104. The average genetic similarity coefficient obtained was 0.06. The dendrogram generated based on SSR marker data revealed the presence of three major clusters, *viz*., A, B and C. The cluster analysis proved that SSR markers utilized in this study could distinguish the different rice genotypes (Fig. 1.). According to parentage, genotypes were

found to cluster together exhibiting their similarity. GR104 a cross between GR101 and Basmati 370 clustered with GR101. Similarly GR-12 a cross between GR-4 and IR-64 and Ashoka 200F a cross between KalingaIII and IR-64 clustered together since they have one common parent in IR-64. This kind of clustering pattern depending upon their pedigree was evident in other crosses also.GR11 and GR4 varieties are sister selections obtained from cross between Zinia-31 and IR-8-246. GR12 is outcome of cross between GR4 and IR-64. So, GR11 and GR12 were clustered together. AAUDR-1 is outcome of cross between Sathi 34-36 and Dadri kolam. So, Sathi 34-36 and AAUDR-1 were clustered together.

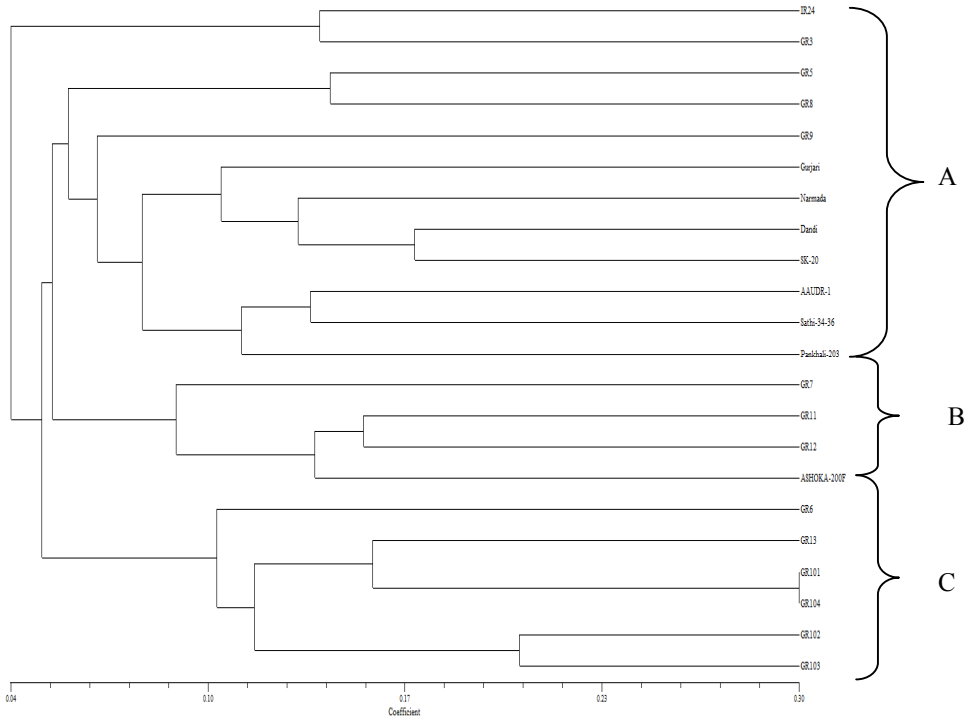

Fig.1. Dendrogram generated by SSR markers using UPGMA based on jaccard's coefficient

Rf genes (Rf 3 and Rf 4) specific SSR marker analysis

The dendrogram generated using pooled SSR loci from genomic regions of *Rf* 3 and *Rf* 4 genes revealed that confirmed fertility restorers viz., GR-101 and GR-104 clustered together along with other cultivars GR-6, GR-13, GR-103, Sathi-34-36, Narmada, GR-102, AAUDR-1 and Pankhali-203. This

indicated that the presence of fertility restorer genes in varieties other than GR-101 and GR-104 cannot be ruled out.

GR-7, which is also a confirmed restorer clubbed with genotypes GR-5, GR-8, GR-9, GR-11, Asokha 200F, Gurjari, Dandi and SK-20 indicating that fertility restorer genes may be harbouring in latter cultivars also. Clustering of genotypes viz., GR-11 and SK-20 (confirmed maintainers) with GR-7 (confirmed restorer) indicated their partial restoration capability. In cluster A, cultivars such as GR-3 and GR-12 clustered with confirmed restorer IR-24.

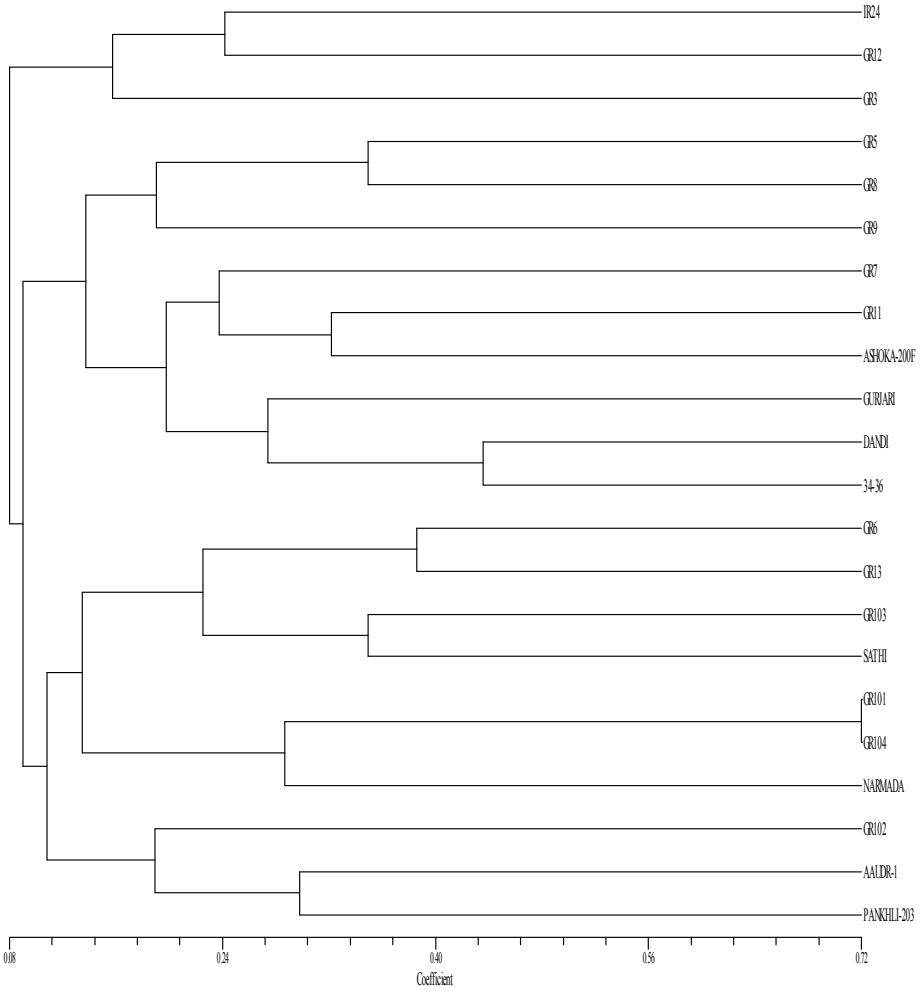

Fig.2. Dendrogram generated by *Rf3* and *Rf4* gene specific SSR markers

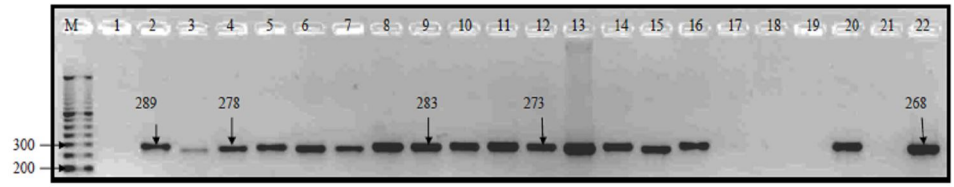

Fig.3 a. SSR profile of *Oryza sativa* L. cultivars produced by primer RM208

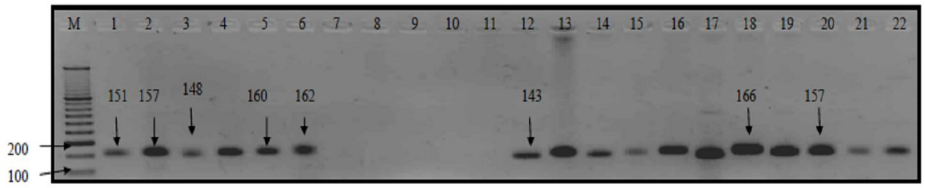

Fig.3 b. SSR profile of *Oryza sativa* L. cultivars produced by primer RM223

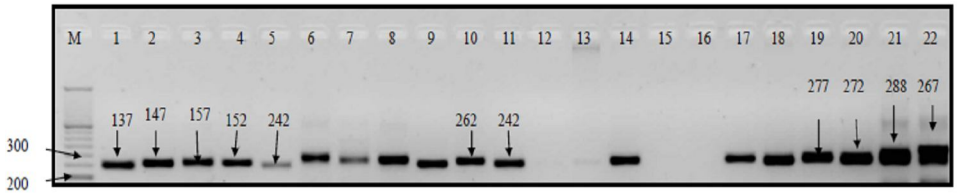

Fig.3 c. SSR profile of *Oryza sativa* L. cultivars produced by primer RM576

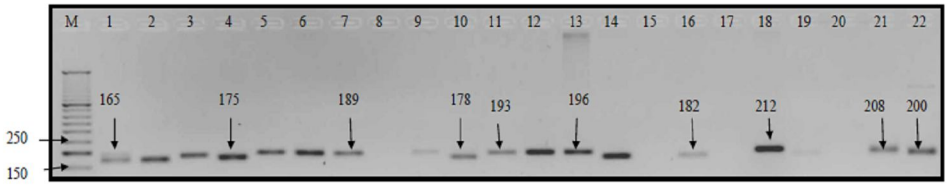

Fig.3 d. SSR profile of *Oryza sativa* L. cultivars produced by primer RM7102

M	50 bp ladder	3	GR5	6	GR8	9	GR12	12	GR102	15	AAUDR	18	Gurjari	21	Sathi 3436
1	IR24	4	GR6	7	GR9	10	GR13	13	GR103	16	Ashoka200F	19	Narmada	22	SK20
2	GR3	5	GR7	8	GR11	11	GR101	14	GR104	17	Dandi	20	Pankhali 203		

Discussion

Microsatellites (SSRs - Simple Sequence Repeats: STRs - Simple Tandem Repeats) are usually the genetic markers of choice for most of the population/conservation genetic and genetic mapping studies. The *Rf3* gene was flanked by the two SSR markers. RM315 and RM443, at distances of 20.7 and

4.4cM, respectively, located on chromosome 1 (Bazrkar *et al.,* 2008, Sheeba *et al*., 2009). RM443 present at the shortest distance, amplified 12 alleles, allelic frequency of it varying from 2.5- 15% percent. The PIC value of it obtained was 0.88. RM315 present at the longest distance, though, it amplified 6 number of alleles. Allelic frequency of it varied from 4.5- 36.3%. The PIC value observed was 0.75. The *Rf4* gene was flanked by RM171, RM6737, RM6100 and RM1108, at distance of 3.2, 1.6, 2.3 and 1.6 cM, respectively (Bazrkar *et al*., 2008, Ahmadikhah *et al.,* 2006). Among these markers the highest allele frequency obtained with RM6100 was 40% percent which has lowest PIC value 0.78. *Rf4* gene was also flanked by the markers viz., RM258, RM3530, RM5359, RM10305, RM10313, RM10318, RM8146, and RM6344 (Khera *et al*., 2006, Bazrkar *et al.,* 2008). Among these markers, the highest allele frequency obtained with RM258 marker was 38% percent with lowest PIC value, 0.74. The highest PIC value obtained was 0.89 with RM10313 marker. For the genetic diversity analysis, it was observed that when the twenty two rice genotypes were analyzed at molecular level using eleven markers, the genotypes 'Gurjari' amplified highest allele length, maximum number of times(4). Similarly for allelic frequency GR102 appeared maximum number of occasions. Lapitan *et al.* (2007) and Mc Couch *et al.* (2002) had also used similar markers for diversity analysis of rice genotypes. For molecular analysis of fertility restorer gene, it was observed that when the 22 rice genotypes were analyzed at molecular level using fourteen markers, the genotype 'GR9' amplified highest allele length, maximum number of times. Similarly for allelic frequency, GR6 and GR13 appeared maximum number of occasions. Ahmadikhah *et al.* (2006), Bazrkar *et al*. (2008), Sheeba *et al*. (2009) and Sattari *et al*. (2009) had also used similar markers for diversity analysis of rice genotypes.

It was observed form this investigation that the SSR markers involved could reveal the latent, diversity present in 22 rice genotypes. Regarding fertility restorer genes, the study revealed that the highest allelic frequencies were observed in genotypes which are non-restorer. Similarly for allele length and allelic frequencies, the restorer failed to amplify maximum number of occasion when screened with different SSR markers.

It was inferred that allelic length may not have any significant correlations with fertility restoration, since the sterility and restoration are controlled by a combination of cytoplasmic and nuclear genes, the expression of restorer genes may be influenced by large number of genetic and epigenetic factors. Exact complimentarity between the primers and genotypes may, to a certain extent help in identifying and characterizing fertility restorer genes.

Conclusion

From this investigation, it was revealed that the molecular markers could expose latent diversity present in rice genotypes. Regarding fertility restorer genes, the highest allelic frequencies were observed in genotypes which were non restorers, similarly for allele length and allele frequencies, the restorers failed to amplify maximum number of occasions when screened with different SSR primers. No relationship between allele length, allele frequency and presence of fertility restorer genes could be established from the study. Fertility restoration ability exhibited by the rice cultivars such as GR101, GR014 and GR7 and maintainers (Partial restorer) *viz*., GR11 and SK-20 indicates that tagging of fertility restorer genes *viz*., *Rf3* and *Rf4* can be accomplished through molecular markers.

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