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Characterization of maintainer and restorer lines for wild abortive cytoplasmic male sterility in indica rice (*Oryza sativa* L.) using pollen fertility and microsatellite (SSR) markers

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Abstract

A narrow genetic base and insufficient numbers of parental lines are major constraints in the development of location-specific hybrid rice varieties. We have studied 4 wild abortive (WA) cytoplasmic male sterile (CMS) lines and 45 pollen parents of diverse origins in order to identify potential restorers and maintainers using test crosses. Pollen viability tests from 85 successful crosses were able to differentiate parental lines into restorers, partial restorers, partial maintainers, and maintainers. Additionally Simple Sequence Repeat (SSR) markers were used to characterize 18 selected entries comprising 8 effective restorers, 4 partial restorers, 2 weak maintainers and 4 complete maintainers. Analysis of divergence patterns based on amplification profiles obtained from 34 SSR markers, allowed differentiation of effective fertility restorers from the rest of the entries comprising partial fertility restorers, weak sterility maintainers and complete sterility maintainers.

Keywords: Hybrid rice, Maintainers and restorers, Pollen patterns, Simple Sequence Repeat (SSR), Wild Abortive Cytoplasmic Male Sterile (WA-CMS).

Introduction

Rice plays a major role in providing human nutrition and food security in developing countries including India. The Green Revolution enabled the rice production to meet the demands of the increasing population and helped many countries to escape from starvation and is currently a major staple food providing more than 65% of caloric intake in many developing countries (Sharma et al., 2013). Since the 1990s, however, rice production has failed to keep pace with population growth. The continued population increase in Asia at the rate of 1.8% per year requires 70% more rice production in 2025 than that in 1995. Moreover, this additional larger quantity of rice has to be produced on limited land, with less water, labour, and chemical resources. Hybrid rice technology has the potential to start to bridge the yield gap and to meet the challenge of increasing rice production while sustaining the natural resource base. Hybrid rice technology exploits the phenomenon of hybrid vigour (heterosis) to increase the yield potential of rice varieties by 15% to 20% over current commercial cultivars (Virmani et al., 1983; Hwa and Yang, 2008). The commercial success of hybrid rice in China has clearly demonstrated the potential of this technology (Matthayatthaworn et al., 2011). CMS is widely used for hybrid seed production. Three line hybrid rice involves the combination of a CMS line, a maintainer line and a restorer line to restore fertility and has proved of considerable value for the development of hybrid varieties.

The traditional approach for identification of maintainer and restorer lines is by observation of pollen fertility and spikelet fertility in test crosses involving CMS lines. In general, identification of different restorer lines for different CMS sources helps in increment of diversity. These lines can be used to diversify the genetic background in order to prevent genetic susceptibility, which arises due to single CMS source. Recently, molecular markers have also been used by several researchers for the tagging of fertility restorer genes and classification of pollen parents into the categories of sterility maintainers and fertility restorers. Microsatellite or simple sequence repeat (SSR) markers have widely been used for the identification of linkage between sterility maintainers and fertility restorers and for determination of genomic location of the two fertility restorer loci known to be involved in fertility restoration of WA type CMS system in rice (Ahmadikhan et al., 2007; Jing et al., 2001; Bazarkar et al., 2008; Sattari et al., 2007; Sheeba et al., 2009; Alavi et al., 2009; Shah et al., 2012; Ghara et al., 2012; Revathi et al., 2013). In the present investigation, an attempt was made to identify sterility maintainers and fertility restorers of WA-CMS and to characterize these lines using SSR markers.

Results

Identification of restorers and maintainers

Experimental materials for the identification of sterility maintainers and fertility restorers comprised test cross progenies derived from combinations involving 4 WA-CMS lines and 45 pollen parents. Using pollen fertility and spikelet fertility as the indices of fertility restoration, the pollen parent of test cross hybrids was categorized as effective restorer, partial restorer, weak maintainer or complete maintainer (Supplemental Table S1). Pollen and spikelet fertility in the test cross progenies ranged from 0% to 97.07% and 0% to

Table 1. List of primers used for amplification of rice genomic DNA in present investigation.

Locus	Chromosome No.	Primer sequence (5'-3')		Repeat motif	Annealing temp. (°c)
		Forward Primer (5'-3')	Reverse Primer (5'-3')		
RM 1	1	GCGAAAACACAATGCAAAAA	GCGTTGGTTGGACCTGAC	$(GA)_{26}$	48
RM 10	7	TTGTCAAGAGGAGGCATCG	CAGAATGGGAAATGGGTCC	$(GA)_{15}$	60
RM 17	12	TGCCCTGTTATTTTCTTCTCTC	GGTGATCCTTTCCCATTTCA	$(GA)_{21}$	51
RM 171	10	AACGCGAGGACACGTACTTAC	ACGAGATACGTACGCCTTTG	$(GATG)_5$	52
RM 201	9	TCGTTTATTACCTACAGTACC	CTACCTCCTTTCTAGACCGATA	$(CT)_{17}$	52
RM 206	11	CCCATGCGTTTAACTATTCT	CGTTCCATCGATCCGTATGG	$(CT)_{21}$	48
RM 216	10	GCATGGCCGATGGTAAAG	TGTATAAAACCACACGGCCA	$(CT)_{18}$	48
RM 228	10	CTGGCCATTAGTCCTTGG	GCTTGCGGCTCTGCTTAC	$(CA)_6(GA)_{36}$	52
RM 234	7	ACAGTATCCAAGGCCCTGG	CACGTGAGACAAAGACGGAG	$(CT)_{25}$	58
RM 247	12	TAGTGCCGATCGATGTAACG	CATATGGTTTTGACAAAGCG	$(CT)_{16}$	50
RM 250	2	GGTTCAAACCAAGCTGATCA	GATGAAGGCCTTCCACGCAG	$(CT)_{15}$	58
RM 258	10	TGCTGTATGTAGCTCGCACC	TGGCCTTTAAAGCTGTCGC	$(GA)_{21}(GGA)_3$	56
RM 263	2	CCCAGGCTAGCTCATGAACC	GCTACGTTTGAGCTACCACG	$(CT)_{34}$	60
RM 315	1	GAGGTACTTCCTCCGTTTCAC	AGTCAGCTCACTGTGCAGTG	$(AT)_4(GT)_{10}$	62
RM 337	8	GTAGGAAAGGAAGGGCAGAG	CGATAGATAGCTAGATGTGGCC	$(CTT)_{4\text{-}19\text{-}}(CTT)_8$	56
RM 407	8	GATTGAGGAGACGAGCCATC	CTTTTTCAGATCTGCGCTCC	$(AG)_{13}$	58
RM 443	1	GATGGTTTTCATCGGCTACG	AGTCCCAGAATGTCGTTTCG	$(GT)_{10}$	52
RM 510	6	AACCGGATTAGTTTCTCGCC	TGAGGACGACGAGCAGATTC	$(GA)_{15}$	58
RM 524	9	TGAAGAGCAGGAACCGTAGG	TCTGATATCGGTTCCTTCGG	$(AT)_{11}$	54
RM 538	5	GGTCGTTGAAGCTTACCAGC	ACAAGCTCTCAAAACTCGCC	$(GA)_{14}$	54
RM 558	12	GAACTCCTCGAACTCGATGC	AGGCATTCAACCTGTTCGAC	$(ATTG)_5$	54
RM 591	10	CTAGCTAGCTGGCACCAGTG	TGGAGTCCGTGTTGTAGTCG	$(AC)_{10}$	58
RM1108	10	GCTCGCGAATCAATCCAC	CTGGATCCTGGACAGACGAG	$(AG)_{12}$	56
RM 3530	1	GTAGATCCGGTCAGCTCCTC	CAAGGAGATTCCCTTCCATG	$(CT)_{39}$	60
RM3873	1	GCTAGCTAGGACCGACATGC	CCTCCTCCTTATCCTCCCTG	$(GA)_{50}$	58
RM 3530	1	GTAGATCCGGTCAGCTCCTC	CAAGGAGATTCCCTTCCATG	(CT)39	60
RM3873	1	GCTAGCTAGGACCGACATGC	CCTCCTCCTTATCCTCCCTG	(GA)50	58
RM 5359	1	CGTGATCTCGTGCATCCC	CCCTCAGGAGCTTCATGAAC	(TC)13	54
RM 5373	10	GGAGATGCTATAGCAGCAGTG	ATTGCTCCTTACCACCTTGC	(TC)13	54
RM 6100	10	TCCTCTACCAGTACCGCACC	GCTGGATCACAGATCATTGC	(CGA)8	54
RM 6344	7	ACACGCCATGGATGATGAC	TGGCATCATCACTTCCTCAC	(GAA)8	52
RM 6737	10	CATTGGGGGTGGATAAAGAG	TATCCTCTACTCCCTCGGCC	(TAT)21	54
RM 7003	12	GGCAGACATACAGCTTATAGGC	TGCAAATGAACCCCTCTAGC	(AAAC)6	54
RM 7241	1	TGCACGGACAGATCAGTTTC	ACTGAACAACACCAAGTGCG	(ATCC)6	52
RM 8146	1	GACTCCTCCAAGTGCAACG	GTAGCTTCCCCACAATGTCA	(AT)22	54
RM 10318	1	TGTCTCACACATTGCACACTTACC	GGCCTAACCCAACACATGTCC	(ACAT)5	56

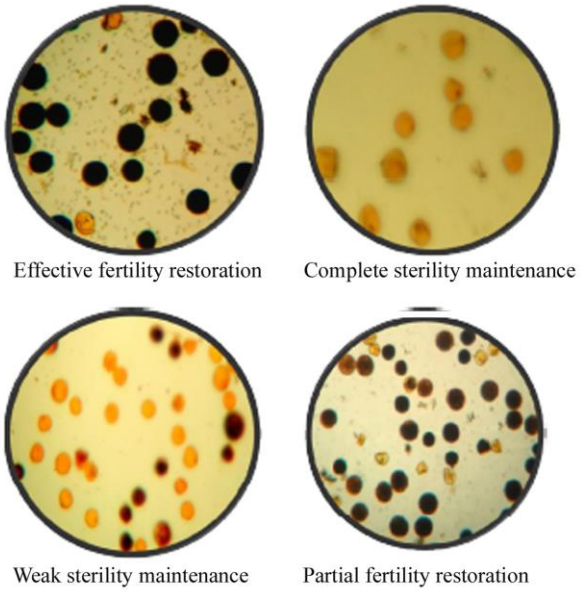

Fig 1. Pollen fertility restoration patterns in test crosses. The harvested panicles were fixed in acetone : alcohol (1:3) solution. Anthers were smeared in solution congaing 0.5% iodine in 2% potassium iodide and examined under light microscope.

99.38%, respectively. The highest pollen fertility was observed in testcross progenies obtained due to cross between RAU1-16-48 (pollen parent) and CMS line 2A (female parent) (Supplemental Table S1). The spikelet fertility was found to be the highest (99.38%) in test cross progenies involving CMS line 5A in combination with pollen parent RAU 670 (Supplemental Table S1). The different categories of pollens and pollen fertility restoration pattern (Fig 1) observed in the test crosses varied with the CMS lines and the pollen parents. The frequency of cross combinations exhibiting complete sterility maintaining behaviour was found to be 23.08, 20.00, 10.00 and 5.26% for the CMS lines 2A, 3A, 4A and 5A, respectively. In some of the entries obtained from the pollen parents of Rajendra Bhagwati, Rajendra Suwasni, Dhanlakshmi and Pusa 1121 yielded male sterile progenies in the cross combinations studied (Supplemental Table S1). In present investigation, all the pollen parents in combination with 5A were identified as effective or partial restorers.

Characterization of restorers and maintainers using Simple Sequence Repeat (SSR) markers

A set of 34 SSRs located on different chromosomes of rice (Table 1) were used to characterize 18 selected restorers and maintainers entries (Supplemental Fig 2; Supplemental Table S2). Out of 34 SSRs, 28 SSRs were able to detect polymorphism except RM250, RM315, RM443, RM510, RM558, and RM7241 SSRs which were found to be monomorphic among the 18 restorers and maintainers entries studied. A total of 266 and 232 alleles were detected at the loci of 34 total and 28 polymorphic SSRs, respectively, studied. The number of alleles per locus generated by each SSR marker varied from 2.5 to 10 alleles, with an average of 5.66 alleles per locus. The highest and lowest number of alleles was detected in the locus generated by RM 263 and RM 3530 marker, respectively. Eleven SSRs showed amplification of more than one locus (Supplemental Table S3). The total alleles identified in the all the 18 entries studied were classified into two categories; (a) unique alleles and (b) shared alleles. A total of 132 unique and 134 shared allelic variants were generated in the form of amplified products by using 34 SSRs. The number of shared alleles per locus ranged from 2 to 7. The maximum polymorphism was observed in RM 538 (71.42%) and minimum in RM7003 (16.66%) SSR marker. These results revealed ample diversity at the molecular level amongst the 18 entries studied.

Polymorphism information content (PIC) analysis

SSR markers were found to be highly informative and polymorphic as evident from its PIC value. The PIC value of each marker, which can be evaluated on the basis of its alleles, varied greatly for all tested SSR loci from 0.53 to 0.94 with an average of 0.76 (Table 2). The highest PIC value was observed for RM 1108 (0.94) whereas, lowest was observed in case of RM 6737 (0.76).

Data analysis and clustering of entries

The data obtained from SSR markers were analyzed as described in Materials and Methods. Considering the bands obtained using all the 34 markers, the similarities coefficient were ranged from 0.0 to 0.551 (Table 3). The dendrogram was generated following unweighted pair group method using arithmetic mean (Fig 2) and the clusters were identified at appropriate phenon level. Considering broad classification of entries, as indicated by dendrogram, all genotypes were divided into two groups (Fig 2). By drawing the phenon line at 25 similarity units and allowing the entries with comparatively more similar pattern for markers to be clustered together, the first multi-genotypic group was further divided into two clusters, whereas the second multi-genotypic group was divided into three clusters (Table 4). Therefore, five clusters were obtained when phenon line was drawn at 25 similarity units. The multi-genotypic cluster A consisted of entries RAU 1-16, RAU 670, RAU 722, RAU 1428-3, PSRM 16 and Rajendra Mahsuri. The di-genotypic cluster B consisted of entries Ranvir Basmati and Sanwal Basmati. The multi-genotypic cluster C accommodated the

Table 2. Analysis of SSRs used for the amplification of genomic DNA extracted from 18 rice entries. The values written in bold represent monomorhic loci.

S. No	Primer	No. of locus amplified	Size of alleles (bp)	No. of alleles	No. of alleles per locus	No. of unique alleles	No. of shared alleles	PP	PIC
1.	RM 1	2	100.00-167.86	12	06	6	6	50.00	0.88
2.	RM 10	1	179.17-213.64	07	07	4	3	57.14	0.78
3.	RM 17	2	178.95-233.33	10	05	6	4	66.66	0.82
4.	RM 171	1	346.67-386.67	06	06	3	3	50.00	0.70
5.	RM 201	1	142.31-340.00	08	08	5	3	62.50	0.81
6.	RM 206	2	142.31-204.55	11	5.5	6	5	54.54	0.84
7.	RM 216	1	142.86-169.23	06	06	2	4	33.33	0.77
8.	RM 228	1	134.62-205.00	09	09	6	3	66.66	0.82
9.	RM 234	1	144.74-184.62	09	09	5	4	55.55	0.85
10.	RM 247	1	150.00-216.67	07	07	3	4	42.85	0.78
11.	RM 250	1	160.00-176.67	06	06	2	4	33.33	0.78
12.	RM 258	1	146.43-195.00	07	07	4	3	57.14	0.76
13.	RM 263	1	175.00-223.08	10	10	6	4	60.00	0.85
14.	RM 315	1	136.36-154.17	04	04	1	3	25.00	0.62
15.	RM 337	3	154.17-445.45	12	04	5	7	41.66	0.85
16.	RM 407	1	183.33-200.00	04	04	1	3	25.00	0.64
17.	RM 443	1	123.81-138.10	06	06	4	2	66.66	0.59
18.	RM 510	1	123.53-138.24	04	04	1	3	25.00	0.69
19.	RM 524	2	206.25-550.00	07	3.5	2	5	28.57	0.56
20.	RM 538	1	272.22-321.43	07	07	5	2	71.42	0.71
21.	RM 558	1	250.00-317.65	08	08	5	3	62.50	0.80
22.	RM 591	2	186.36-337.50	13	7.5	8	5	61.53	0.84
23.	RM1108	1	131.58-150.00	08	08	2	6	25.00	0.94
24.	RM 3530	2	161.54-205.50	05	2.5	2	3	40.00	0.67
25.	RM3873	3	146.67-400.00	13	4.3	6	7	46.15	0.93
26.	RM 5359	1	200.00-225.00	05	05	2	3	40.00	0.62
27.	RM 5373	1	126.67-146.67	07	07	3	4	42.85	0.82
28.	RM 6100	2	156.25-200.00	07	3.5	2	5	28.57	0.80
29.	RM 6344	1	119.44-153.33	09	09	6	3	66.66	0.81
30.	RM 6737	1	155.56-250.00	06	06	4	2	66.66	0.53
31.	RM 7003	2	100.00-117.65	06	03	1	5	16.66	0.71
32.	RM 7241	1	136.67-163.33	06	06	2	4	33.33	0.79
33.	RM 8146	2	108.33-260.00	13	6.5	9	4	69.23	0.79
34.	RM 10318	1	200.00-228.57	08	08	3	5	37.50	0.83
	Total	47	-	266	-	132	134	-	25.97
	Average								0.76

PP: Polymorphism per cent; PIC: Polymorphism information content

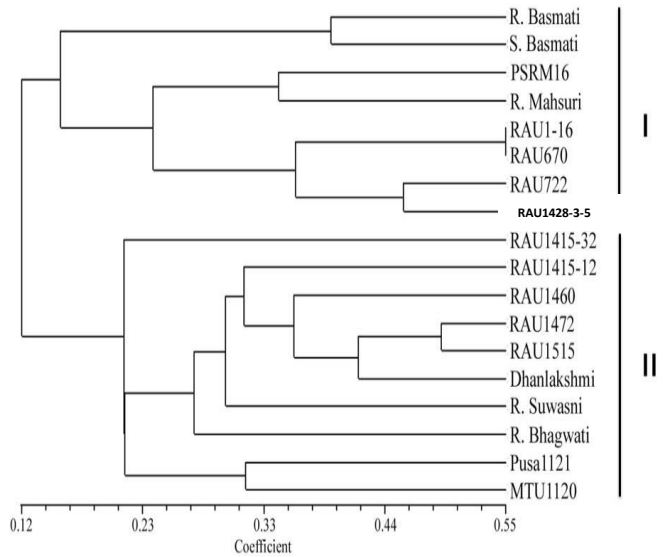

Fig 2. Dendrogram based on Dice similarity coefficient among 18 rice genotypes evaluated using 34 SSR markers. All genotypes were divided into two major groups.

entries RAU 1472, RAU 1515, Dhanlakshmi, RAU 1460, RAU 1415-12, Rajendra Suwasni and Rajendra Bhagwati. The di-genotypic cluster D consisted of entries Pusa 1121 and MTU 1120. The mono-genotypic cluster E included the entry RAU1415-32. Keeping 50 similarity units as the cut-off point, when phenon line was drawn to discriminate the genotypes, the clusters A and D were further dissociated into two sub-clusters, whereas the cluster C was further dissociated into four sub-clusters (Table 4). The cluster A was dissociated into two sub-clusters; one di-genotypic sub-cluster AII and the other tetra-genotypic sub-cluster AI (Table 4). The tetra-genotypic sub-cluster AI had entries RAU 1-16, RAU 670, RAU 722 and RAU 1428-3, whereas di-genotypic sub-cluster AII accommodated PSRM 16 and Rajendra Mahsuri (Table 4). The clusters B, D and E did not dissociate at 50 similarity units. Clustering pattern further indicated that sub-clusters AI, AII, B, and CI were further dissociated into sub-sub-clusters by drawing the phenon line at 75 similarity units as cut off point. The sub-cluster AI was dissociated into two di-genotypic sub-sub-clusters; AIa which included RAU 1-16 alongwith RAU 670 and AIb which accommodated RAU 722 alongwith RAU 1428-3. The sub-cluster AII was dissociated into two mono-genotypic sub-sub-clusters AIIa and AIIb accommodating PSRM 16-48 and Rajendra Mahsuri, respectively (Table 4). Similarly, the cluster B and the sub-cluster CI dissociated into two and three mono-genotypic sub-sub-clusters, respectively (Table 4).

Principal component analysis (PCA)

Associations among the 18 entries were also examined with PCA (Fig 3). PCA showed the distribution and grouping of rice entries. The two-dimensional plot of the 18 rice entries confirmed the clustering pattern obtained by the cluster analysis.

Discussion

The identification of maintainers and restorers is fundamental for the commercial exploitation of heterosis breeding programme using cytoplasmic male sterility (CMS) system (Rosamma and Vijayakumar 2005; Sharma et al., 2012). Restorers for different cytosterile sources will increase the cytoplasmic diversification, which in turn can prevent genetic vulnerability due to the use of single CMS source. While a large number of restorers have been identified for the wild abortive (WA) CMS lines (Virmani and Edwards, 1983; Rosamma and Vijayakumar 2005; Sharma et al., 2012), the identification of effective restorers for locally available high yielding rice genotype is still limiting. Therefore, the present investigation was undertaken to identify maintainers and restorers for wild abortive cytoplasmic male sterility among the local and high yielding rice (*Oryza sativa* L.) varieties of Bihar. Staining patterns and shapes of pollen grains in genotypes of rice possessing male sterility-inducing cytoplasm and sterility-maintaining nuclear gene(s) are known to be influenced by the developmental stage at which pollen abortion occurs and these developmental stages are related to nuclear stages (Chaudhary et al., 1981; Elkonin and Tsvetova 2012). Based on staining pattern obtained from the different test crosses made, the pollen parents are classified as effective restorers, partial restorers, weak maintainers and complete maintainers. Pollen fertility in test cross progenies were found strongly correlated (r=0.952) with spikelet fertility, indicating that either pollen or spikelet fertility could be used as a criterion to classify pollen parents as maintainers or restorers. Therefore, the extent of spikelet fertility was used for the classification of pollen parents used in test crosses as previously described (Govinda and Virmani, 1988; Joshi et al., 2007). Our study is in agreement with Joshi et al. (2007) in that highly significant positive correlation was found between stained round fertile (SRF) pollen and spikelet fertility. However, Joshi et al. (2003) reported that pollen's susceptibility to staining with I-KI solution does not correlate with spikelet fertility. This may be due to the ability of single fertile pollen to fertilize a spikelet (Joshi et al., 2003). Effective restorers identified on the basis of pollen fertility were characterized by relatively low spikelet fertility restoration in some of the test crosses made in this study. Some of the cross combinations (< 60%) showed pollen fertility higher than 60% and exhibited more than 80% spikelet fertility. Since, high seed yield of hybrid lines depends largely on high spikelet fertility (Bagheri and Babaeian-Jelodar 2011), and recording observation on pollen fertility is more time consuming, spikelet fertility could alone be considered as the basis for classification of pollen parents into maintainers and restorers. In present study the pollen parents, RAU 1415-12, RAU 1415-32, RAU 1460-4 and RAU 1472-4 were identified as good partial restorers for one or more of the CMS lines. Pollen parents from Rajendra Bhagwati, Rajendra Suwasni, Dhanlakshmi and Pusa 1121 yielded completely male sterile progenies in the test crosses. Therefore, these pollen parents were identified as complete sterility maintainers of WA type cytosterility system. However, in this study, pollen parents identified as restorers or partial restorers of one CMS line showed partial restoration or weak maintaining ability in crosses with other iso-cytoplasmic CMS lines. Differential reaction of the same genotype in crosses with different CMS lines carrying the WA type cytoplasm has also been reported by other researchers (Hariprasanna et al., 2005; Bisne et al., 2005; Salgotra et al., 2007; Sabar et al., 2007; Jayasudha et al., 2010; Umadevi et al., 2010). The differential expression of fertility restoration may be due to the influence of the genetic back ground of the female parent (Hossain et al., 2010). Wilson (1998) interpreted this phenomenon as being the result of variation in the number of sterility genes and/or effectiveness among CMS lines or the fertility genes that act in a complementary or additive fashion with restorer genes. Excess sterility genes could act as inhibitors of pollen fertility restoration in the F_1 generation. Incomplete fertility restoration of WA type CMS lines by some established restorers has also been attributed to the existence of inhibitory genes (Govinda and Virmani, 1988) or hybrid sterility genes in the CMS lines. Pradhan and Jachuck (1998) suggested that the partial restoration/effective maintenance in some cases must be due to heterozygous fertility restoration genes. The diversity within the CMS lines derived from WA source and differential interaction of both the cytoplasm as well as the nuclear background of female parent with the pollen parent (Kumary and Mahadevappa, 1998) or the effect of minor or modifier genes present in the pollinator (Ganesan et al., 1998) could also result in differential fertility restoration. Moreover, penetrance and/ expressivity of the weaker restorer gene have also been found responsible for variation in different genetic background of the female parent (Govinda and Virmani, 1988; Singh et al., 2005). The use of molecular marker technology provides a powerful tool for genetic characterization of entries at the molecular level, in addition to assessment of genetic variability, elucidation of genetic relationships and measurement of genetic diversity within and among species (Chakaravarthi and Naravaneni,

Table 3. Estimates of 34 SSR primer pairs based Dice's similarity coefficients among 18 rice entries used in the present study

	R.Basmati	S.Basmati	PSRM16	R.Mahsuri	RAU 1-16	RAU 670	RAU 722	RAU 1428-3	RAU 1415-32	RAU 1415-12	RAU 1460	RAU 1472	R.Suwasini	R.Bhagti	RAU1515	Dhanlakshmi	Pusa1121
S. Basmati	0.395																
PSRM16	0.222	0.278															
R. Mahsuri	0.192	0.219	0.348														
RAU1-16	0.111	0.250	0.353	0.348													
RAU670	0.137	0.137	0.232	0.257	0.551												
RAU722	0.051	0.128	0.135	0.213	0.351	0.400											
RAU1428-3	0.028	0.083	0.147	0.203	0.353	0.348	0.459										
RAU1415-32	0.080	0.053	0.084	0.056	0.113	0.139	0.156	0.282									
RAU1415-12	0.111	0.111	0.088	0.058	0.147	0.145	0.162	0.294	0.282								
RAU1460	0.056	0.056	0.088	0.116	0.147	0.116	0.297	0.265	0.225	0.294							
RAU1472	0.110	0.082	0.087	0.057	0.116	0.143	0.187	0.232	0.194	0.435	0.406						
R. Suwasini	0.111	0.056	0.059	0.174	0.029	0.000	0.108	0.206	0.169	0.294	0.323	0.406					
R. Bhagti	0.080	0.027	0.028	0.056	0.169	0.139	0.156	0.225	0.270	0.225	0.310	0.222	0.282				
RAU1515	0.079	0.053	0.056	0.027	0.111	0.164	0.231	0.250	0.133	0.278	0.389	0.493	0.278	0.347			
Dhanlakshmi	0.082	0.055	0.087	0.086	0.087	0.086	0.133	0.172	0.194	0.261	0.290	0.400	0.203	0.250	0.438		
Pusa1121	0.082	0.055	0.058	0.143	0.087	0.171	0.187	0.174	0.167	0.203	0.261	0.143	0.087	0.194	0.219	0.229	
MTU1120	0.056	0.056	0.000	0.087	0.118	0.087	0.243	0.294	0.253	0.147	0.265	0.232	0.147	0.253	0.278	0.290	0.319

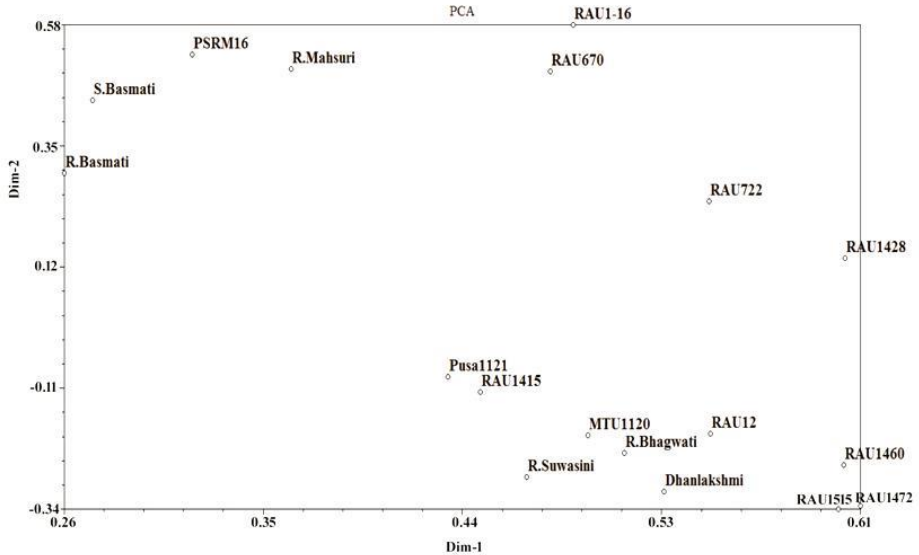

Fig 3. Principle component analysis of 18 rice entries based on 34 SSR marker's data.

Table 4. Composition of clusters based on similarity coefficient in numerical taxonomic approach of cluster analysis.

Clusters identified at different phenon levels			Entries included in each clusters
25	50	75	
A (6)	AI (4)	AIa (2)	RAU 1-16, RAU 670
		AIb (2)	RAU 722, RAU 1428-3
	AII (2)	AIIa (1)	PSRM 16
		AIIb (1)	RajendraMahsuri
B (2)	B (2)	BI (1)	Ranvir Basmati
		BII (1)	Sanwal Basmati
C (7)	CI (4)	CIa (2)	RAU 1472, RAU 1515
		CIb (1)	Dhanlakshmi
		CIc (1)	RAU 1460
	CII (1)	CII (1)	RAU 1415-12
	CIII (1)	CIII (1)	RajendraSuwasni
	CIV (1)	CIV (1)	RajendraBhagwati
D (2)	DI (1)	DI (1)	Pusa 1121
	DII (1)	DII (1)	MTU 1120
E (1)	E (1)	E (1)	RAU 1415-32

2006). SSR markers has widely been used to differentiate rice genotypes (Joshi and Behera, 2006; Brondani et al., 2006; Lapitan et al., 2007; Pervaiz et al., 2009; Rabbani et al., 2010; Vanaza et al., 2010; Pervaiz et al., 2010; Singh et al., 2011). In present investigation, 34 SSR markers has been used to characterize 18 entries comprising effective restorers, partial restorers, weak maintainers and complete maintainers of WA type CMS in rice. Out of 34 SSRs used 23 SSRs and 9 SSRs amplified single and more than one band, respectively, in all the entries studied. Similar results were also reported previously (McCouch et al., 2002). Nine SSR markers mentioned in Supplemental Table S2 have also been reported to amplify simple sequence repeats located on different chromosomes (McCouch et al., 2002). The two-dimensional plot generated by PCA analysis also confirms the cluster analysis results. PCA analysis was used previously to validate and or eliminate redundancy in the dataset (Haehemi-Petroudi et al., 2010; Rathi et al., 2014). These results clearly indicated that a considerably greater extent of genetic variability exists among different entries under evaluation. These SSRs have also been used for classification of restorers from maintainers in the studies conducted earlier (Ahmadikhan et al., 2007; Jing et al., 2001; Bazarkar et al., 2008; Sheeba et al., 2009; Shah et al., 2012). Some of these primers in combinations with other SSRs have also been used in earlier studies for classification of fertility restorers from non-restorers (Alavi et al., 2009; Ghara et al., 2012). The level of polymorphism exhibited by each of the SSR marker was further assessed by calculating PIC, which reflects allele diversity and frequency of the markers among the entries. The PIC values observed in the present study are comparable to previous reports (Pervaiz et al., 2009; Lapitan et al., 2007). The mean PIC value obtained in the present study was found to be higher compared to earlier report (Joshi and Behera, 2006), who reported the value as 0.447 and 0.540 per locus. The average PIC value of 0.764, which was obtained in this study, confirms that the SSR markers utilized for molecular characterization of entries under evaluation were, in general, highly informative. The markers utilized in the present study were sufficient for discrimination and unambiguous classification of effective fertility restorers from partial fertility restorers, weak sterility maintainers and complete sterility maintainers.

Materials and Methods

Plant materials and test cross

The experiment for identification of sterility maintainers and fertility restorers of wild abortive type CMS lines of rice included in the present study was conducted at the research farm of Rajendra Agricultural University, Pusa, Bihar. Forty five pollen parents (Supplemental Table 1) were crossed with cytoplasmic male sterile (CMS) lines Pusa 6A (2A), PURI-95-17A (3A), PMS-17A (4A) and PMS-10A (5A) to produce F1 seeds. Each F_1 and the corresponding pollen parent were grown side by side and CMS lines were grown in isolation. Uniform agronomic practices were followed while raising the crop. The 85 test cross combinations were made using selected F_1 with 4 WA-CMS lines (Supplemental Table 1).

Pollen staining and spikelet fertility

The test crosses were evaluated on the basis of pollen and spikelet fertility during *kharif*, 2011 and *kharif*, 2012 for identification of fertility restorers and sterility maintainers. Each test cross was raised in 3 rows following the standard spacing between plant to plant (15 cm) and row to row (20 cm). At the time of flowering, panicles from 3 randomly chosen plants in each of the test cross combination were collected and fixed in acetone alcohol (1:3) solution. Anthers of 3 randomly taken spikelets representing lower middle and top portion of the panicles were smeared in solution congaing 0.5% iodine in 2% potassium iodide and examined under light microscope. The pollen grains were classified as Unstained Withered Sterile (UWS), Unstained Spherical Sterile (USS), Stained Round Sterile (SRS) and Stained Round Fertile (SRF) following the procedure as used earlier by Chaudhury et al. (1981). Only SRF pollen grains were classified as fertile. The observation on pollen fertility was recorded by counting the number of fertile pollen grains in percentage of the total number of pollen grains examined. Based on pollen fertility, the pollen parents of the test cross progenies were classified of pollen parents of the test crosses as probable restorers (>60% fertility), partial restorers (31-60% fertility), weak maintainers (1-30% fertiltity) and complete maintainers (<1% fertility) following the procedure as used by earlier worker (Govinda and Virmani, 1988).
At the time of harvesting, 05 bagged panicles including one from the main culm were harvested from randomly chosen plants in each of the test crosses at maturity. Out of these, five panicles per test cross were randomly taken for recording observation on spikelet fertility. At maturity, the bagged panicles were examined for seed set. The number of filled spikelets and the total number of spikelets were counted and spikelet fertility was expressed as the number of filled spikelets in percentage of the total number of spikelets. Based on spikelet fertility, Classification of the pollen parents of the test crosses as effective restorers (>80% fertility), partial restorers (31-80%fertility), weak maintainers (1-30% fertility) and complete maintainers (<1%fertility) following the method as suggested by earlier worker (Govinda and Virmani, 1988).

DNA isolation

Eighteen rice genotypes (Supplemental Table S2), comprising A (cytoplasmic male sterile line), B (maintainer) and R (restorer) lines were used all genotype were germinated by placing seeds over two layer of wet filter paper and grown for 15 days in the petridishes at room temperature (RT). Total genomic DNA was extracted from young leaves using CTAB (Cetyl Trimethyl Ammonium Bromide method) (Saghai - Maroof, 1984). The quality and quantity of extracted DNA were determined spectrophotometrically by taking absorbance at 260 nm and 280 nm (Varian Cary 50 Spectrophotometer). The extracted DNA samples were diluted using $T_{10}E_1$ buffer to get the working concentration of 50ng/µl. The diluted DNA samples were used for SSR analysis.

Selection of primers

Thirty four microsatellite primer pairs were used in study for characterization of fertility restorers and sterility maintainers. These primers covering all 12 chromosomes were selected for the molecular analysis on the basis of published rice microsatellite frame work map (Panaud et al., 1996). These primers pairs were selected randomly between the previously reported SSR loci and the target Rf gene. A total 34 SSR markers were selected based on published literatures for analysis (Table 1) (Ahmadikhan et al., 2007; Jing et al., 2001; Bazarkar et al., 2008; Sheeba et al., 2009; Shah et al., 2012). The original source, repeat motifs, primer sequences and chromosomal positions for these markers can be found in the rice genome database (http://www.gramene.org).

SSR marker analysis

For SSR analysis PCR conditions were optimized as described previously (Panaud et al., 1996) with minor modifications. PCR amplification reaction were conducted in 15 µl reaction volume containing 50ng of template DNA, 1X PCR buffer, 200 µM each deoxynucleotide triphosphate (dNTPs), 2 mM of $MgCl_2$, 0.4 µM of each forward and reverse primer and 1 unit Taq DNA polymerase (Fermentas). The PCR amplification was carried out using a thermocycler (Biometra) with an initial denaturation step of 94°C for 5 min, followed by 35 cycles of denaturation (1 min at 94°C), annealing depending on the primers used (2 min at 48-60°C), and extension (2 min at 72°C). After the last cycle, a final extension was carried out for 10 min at 72°C. The reproducibility of amplification products was checked twice for each primer. Amplified products were resolved by electrophoresis in 2% agarose gel containing 0.5 µg/ml ethidium bromides. A 50bp DNA ladder (Gene ruler, Fermentas) was used as size marker to compare the molecular weights of amplified products. Electrophoresis was carried out at 100V using electrophoresis system (CBI Scientific) for 2 hrs. After electrophoresis the gels were documented using a Gel documentation system (Alpha Innotech).

Allele scoring and data analysis

Clear and distinct bands amplified by SSR marker were scored for presence (1) and absence (0) of the corresponding bands among the genotype. The data were entered into a binary matrix and subsequently analyzed using simqual- sub-program of software NTSYS-pc version 2.1 (Rohlf 2000). Further, similarity matrix data (Table 3) was used to construct dendrogram by using Sequential Agglomerative Hierarchical Nesting (SAHN) based on unweighted pair-group method with an arithmetic average (UPGMA) to inter genetic relationship and phylogeny among varieties using software NTSYS-pc version 2.1 (Rohlf 2000).

Principal component analysis

Principal component analysis (PCA) was carried out to obtain a two-dimenional plot, in order to represent the diversity among the restorers and maintainers. It was generated using the EIGEN programme of NTSYS-pc software 2.1 (Rohlf, 2000).

Conclusion

The findings of present study evident that fertility restoration reaction of the genotypes varies with genetic background of CMS lines. The identified maintainers and restorers are locally adopted. The identified maintainers namely Rajendra Suwasni, Rajendra Bhagwati, Pusa1121 and Dhanlaxmi can be developed as new members in CMS family by repeated back cross breeding. The identified restorer lines were Ranvir Basmati, Sanwal Basmati, PSRM16-48, Rajendra Mahsuri,

RAU1-16, RAU722, RAU1428 and RAU670. The identified restorer lines can be used as pollen parent in developing new commercial hybrid variety.

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