# Validation Of Microsatellite Markers For Discrimination Of Sterility Maintainers And Fertility Restorers Of Wild Abortive Cytoplasmic Male Sterility In Rice

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**Abstract**

**Use of cytoplasmic male sterility and fertility restoration system greatly facilitates large scale commercial production of hybrid seed in rice. Traditional approach for identification of fertility restorers is based on assessment of pollen fertility and spikelet fertility in test crosses. Genomic markers assisted isolation of sterility maintainers and fertility restorers appears to be more efficient approach. The present investigation was conducted to determine the usefulness of microsatellite markers for differentiation of sterility maintainers and fertility restorers of cytoplasmic male sterility system in rice. Experimental materials of the present study comprised a set of six sterility maintainers and six fertility restorers of wild abortive type cytoplasmic male sterility. Simple sequence repeats based genetic polymorphism was examined by employing a set of eight sequence tagged microsatellite primer pairs for amplification of targeted genomic regions in these sterility maintainers and fertility restorer lines and the two bulk samples made separately for maintainers and restorers by mixing equal amount of genomic template isolated from different entries belonging to these two groups. Computational analysis of amplification profiles led to the validation of four microsatellite primer pairs, which allowed unambiguous differentiation of six sterility maintainers from six fertility restorers. Experimental results demonstrated that these four microsatellite primer pairs, namely, RM 315, RM 6100, RM 258 and RM 443, could be further utilized for screening of large number of restorer and non-restorer lines and identification of effective fertility restorers of wild abortive type cytoplasmic male sterility system. Among the remaining four primer pairs utilized during molecular profiling, RM 116 and RM 3873 also discriminated the fertility restorers from sterility maintainers, but the differential amplification patterns were not easily and conveniently distinguishable.**

**Key words**: Rice, sterility maintainers, fertility restorers, microsatellite, wild abortive

## Introduction

Tropical rice growing countries essentially need to step up their rice production because of burgeoning population and decreasing land and other resources. Among the various technological options contemplated for increasing rice production with no additional land available for cultivation, large scale adoption of hybrid rice technology is considered as practically feasible and technologically sustainable option to break the existing yield ceiling and enhance rice productivity (Mishra *et al.*, 2003; Sheeba *et al.*, 2009). The success of hybrid rice technology primarily depends on the production and timely supply of genetically homogenous seeds to farmers. Exploitation of cytoplasmic male sterility combined with a fertility restoration system has been successfully demonstrated to be the most efficient genetic tool to exploit

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hybrid vigor on a commercial scale in a self pollinated crop like rice.

Cytoplasmic male sterility is caused by lesion or rearrangement of mitochondrial genome resulting in its inability to produce functional pollen. But its fertility can be restored by nuclear genes governing fertility restoration. Using this system, it is convenient to allow the stigma cross-pollination from other plants to generate heterozygous seeds and to create the possibility of scaling up the production of hybrid seeds. Until now, a variety of sources for male sterility inducing cytoplasm and other male sterile germplasm have been discovered and used. The male sterility inducing cytoplasm induces the cytoplasmic male sterility through interaction with the cell nucleus. At present, the hybrid seed production in rice is primarily based on three-line approach, which involves a cytoplasmic male sterile line (A line), its iso-nuclear maintainer line (B line) and a genetically diverse restorer line (R line). The fertility of male sterile line is restored by fertility restorer (*Rf*) gene associated with nuclear genes, mostly encoding penta-tricopeptide repeat proteins (Hanson and Bentolila, 2004). The most essential requirement in the development of hybrid rice is identification of effective fertility restorers that can ensure complete spikelet fertility and high degree of heterosis in traits of economic importance.

Traditional approach for identification of maintainer and restorer lines is cumbersome as it essentially requires analysis of pollen and spikelet fertility in test crosses for the classification of pollen parents of respective test crosses as sterility maintainers or fertility restorers (Kumar *et al*., 2002). Among several types of male sterility inducing cytoplasm discovered in rice, wild abortive cytoplasmic source characterized by sporophytic abortion and formation of typical abortive pollen (Sattari *et al*., 2008) is widely deployed for commercial hybrid seed production. The fertility restoration of this system is considered to be controlled by different *Rf* loci in different restorer lines. However, the involvement of two genes (*Rf3* and *Rf4*) appears to be consistent in restoring the fertility across most of the studies (Zhang *et al*., 1997; Tan *et al*. 1998; Jing *et al*., 2001; Zhang *et al*., 2002; Mishra *et al*., 2003; Ahmadikhah and Karlov 2006; Ahmadikhah *et al*., 2007; Sheeba *et al*., 2009; Cai *et al*., 2013; Chen *et al*., 2017). Using microsatellite markers, both *Rf3* and *Rf4* loci with their chromosomal locations have been identified in different donors and pollen parents (Kumar *et al*., 2015; Bhati *et al*., 2018; Kumar *et al*., 2018) have been classified into the categories of sterility maintainers and fertility restorers. Keeping into consideration the usefulness of microsatellite markers for molecular characterization in rice, the present investigation was carried out to survey the microsatellite markers based polymorphism amongst some sterility maintainers and fertility restorers of wild abortive cytosterility system in rice and to validate the utility of these microsatellite markers for differentiation of sterility maintainer and fertility restorer lines

**Materials and methods**

Experimental materials comprised six sterility maintainers and six fertility restorers of wild abortive type cytoplasmic male sterility system. The seeds of these six sterility maintainers, namely, Faizabad B (B1), Pusa 6B (B2), UPR I-95-17B (B3), PMS 10B (B4), IR 79125B (B5) and IR 80156B (B6) along with six fertility restorers, namely, PRR-78

(R1), RAU 722 (R2), Ranvir Basmati (R3), Sanwal Basmati (R4), RAU 670 (R5) and PSRM 16-48 (R6) were planted in the pots. The immature leaves were collected separately from each entry two weeks after germination and utilized for the extraction of templates (Doyle and Doyle, 1990) to be utilized during primer directed amplification. Employing a panel of eight fertility restoration related microsatellite primer pairs (Table 1), genotypic level polymorphism was surveyed by analysis of amplification profiles of six sterility maintainers, six fertility restorers and the two bulks made separately for maintainers (MB) and restorers (RB) by mixing the equal amount of genomic DNA sample isolated from each of the entries belonging to these two groups. Polymerase chain reaction was carried out in 20 µl reaction volume containing 5.3 µl water (protease and nuclease free), 3.0 µl 5X polymerase buffer (with 10 mM $MgCl_2$), 3 µl dNTPs mix (1 mM), 1.4 µl $MgCl_2$ (10 mM), 1.4 µl primer F (5 µM), 1.4 µl primer R (5 µM), 1 unit Taq polymerase and 4 µl DNA template. The amplification reaction was performed in a thermal cycler keeping initial denaturation at 94º C for 4 min, 30 cycles of denaturation at 94º C for 1 min, primer annealing at 48-62º C (varied with different primers) for 1 min and extension at $72^0$ C for 2 min followed by final extension at $72^0$ C for 10 min and cooling at $4^0$ C. The products of amplification reaction were resolved by agarose (2%) gel electrophoresis and then visualized under gel documentation system. Molecular size of amplified products was determined in relation to the size of markers in ladder (50 bp; Fermentas) with the help of gel reader (Alpha View Gel Reader).

All the entries under evaluation were scored for the presence and absence of the bands and the data were entered into binary matrix as discrete variables. Genetic similarity (Dice 1945) among entries was analyzed for pair-wise comparisons based on the proportions of shared bands produced by the primers as *Similarity coefficient* = $2a/(2a+b+c)$, where, $a$, $b$ and $c$ represent number of bands shared between $J^{th}$ and $K^{th}$ genotypes, number of bands present in $J^{th}$ genotype but absent in $K^{th}$ genotype and number of bands absent in $J^{th}$ genotype but present in $K^{th}$ genotype, respectively. Sequential agglomerative hierarchical non-overlapping (SAHN) clustering approach was adopted for tree building during cluster analysis and the dendrogram was obtained by un-weighted pair-group method using arithmetic mean (UPGMA). Cluster analysis was performed with the help of the software NTSYS-pc (Rohlf 2000). Principal coordinate analysis was also conducted to obtain a two-dimensional ordination of the microsatellite primers specific genetic profiles of the fourteen entries subjected to molecular characterization.

**Results and discussion**

Differential level of polymorphism was revealed among the fourteen entries including six sterility maintainers, six fertility restorers and two bulk samples under evaluation in the present investigation and the polymorphism was recognized in the form of variation in respect of position of bands. Appearance of bands at different positions on the gel revealed differential migration due to differences in overall size of the products generated from amplification of primer specific region of the genome. Altogether 23 allelic variants were detected with an average of 2.8 alleles per locus. The number of alleles per locus ranged from two in the cases of RM 258, RM 315, RM

443 and RM 6100 to four in the cases of RM 171, RM 1108 and RM 3873. All the primers generated single but polymorphic amplified products in combination with all the entries subjected to molecular profiling.

The overall size of amplified products approximately ranged from 300 bp to 350 bp, 150 bp to 162 bp, 153 bp to 165 bp, 140 bp to 150 bp, 150 bp to 175 bp, 133 bp to 162 bp, 197.06 bp to 211.54 bp and 144 bp to 168 bp corresponding to the primer pairs RM 171, RM 216, RM 258, RM 315, RM 443, RM 1108, RM 3873 and RM 6100, respectively. Among the primer pairs yielding more than two types of amplified products, RM 216 generated two types of amplified products in different combinations with sterility maintainers and only one type of amplified product in the cases of fertility restorers, whereas RM 3873 generated two types of amplified products in combinations with sterility maintainers as well as fertility restorers. Remaining two primer pairs, namely, RM 171 and RM 1108 produced four types of amplified products without exhibiting any specificity in respect of molecular size of the product and sterility maintaining or fertility restoring behavior of the entry in question.

Hierarchical classification pattern of the entries, as reveled by similarity coefficients based dendrogram, was observed to be unambiguously related to sterility maintaining or fertility restoring ability of the entries. A perusal of dendrogram clearly reflects that the entries were basically divided into two multi-genotypic groups (Fig. 1) amongst which one multi-genotypic group (B) consisted of six sterility maintaining entries, whereas the second multi genotypic group (R) included six fertility restoring entries. Furthermore, the bulk samples made from sterility maintainers (MB) and fertility restorers (RB) were accommodated into the former and later groups, respectively. Apparently therefore, the panel of eight primer pairs utilized for targeted amplification of genomic regions was completely effective in accurately discriminating the entries and samples in relation to their fertility restoration ability. Principal coordinate analysis based inferences derived from spatial distribution pattern of the entries along the two principal axes in the two dimensional plot of eight fertility restoration related microsatellite primer pairs dependent genetic profiles completely supported the results derived from the similarity coefficients based hierarchical classification patter of entries (Fig. 2). Sterility maintainers and fertility restorers were distinctly divided into different genotypic groups without any intermixing. Using some of these primers in combinations with other primer pairs, similar results have been obtained in earlier studies conducted for classification of fertility restorers from non-restorers (Alavi *et al*. 2009; Ghara *et al*. 2012, Kumar *et al*., 2015; Kumar *et al*., 2018).

Among the eight simple sequence repeat primer pairs utilized, four primer pairs, namely, RM 258, RM 315, RM 443 and RM 6100, were found to yield only two allelic variants of the amplified product per primer pair, differentiating very clearly the sterility maintainer lines from fertility restorers. The amplification profile of all sterility maintainers generated by using these four primer pairs was completely similar to the amplification pattern visualized in the case of bulk sample prepared by mixing the template isolated from each of the six sterility maintainers. Similarly,

amplification profile of all fertility restorers generated by using these four primer pairs exactly corresponded to the amplification pattern visualized in the case of bulk sample prepared by mixing the template isolated from each of the six fertility restorers (Sharma and Nidhi, 2014).

Experimental analysis performed for the presence of *Rf3* gene in the fertility restorers under evaluation in the present investigation using the three microsatellite markers, namely, RM315, RM443 and RM 3873, which were earlier reported to be linked to this gene (Bazrkar *et al*., 2008; Alavi *et al*., 2009; Sheeba *et al*., 2009), led to validation of RM 315 and RM 443. The marker RM 315 could clearly distinguish the sterility maintainers from fertility restorers by generating an allele of 140 bp in case of all the six sterility maintainers and an allele of 150 bp in case of all the six fertility restorers. Similarly, RM 443 generated an allelic variant of 150 bp in cases of all the six sterility maintainers and an allele of 175 bp in cases of all the six fertility restorers (Fig. 3). The primer RM 3873 was also able to differentiate sterility maintainers and fertility restorers by generating amplified products of 197 and 203 bp in the former, whereas 207 and 211 bp in the latter. However, the polymorphism was not easily recognizable due to smaller differences in the molecular size of the amplified products.

Molecular profiling with RM 171, RM 216, RM 258, RM 1108 and RM 6100 to ascertain the presence of *Rf4* gene in fertility restorers resulted in validation of the usefulness of RM 258 and RM 6100, since these two markers amplified two alleles each. The marker RM 258 could clearly distinguish the sterility maintainers from fertility restorers by generating an allele of 153 bp in the cases of all the six sterility maintainers, whereas an allele of 165 bp in the cases of all the six fertility restorers. Similarly, RM6100 produced allelic variants of about 144 bp and 168 bp in the cases of sterility maintainers and fertility restorers, respectively (Fig. 3). Polymorphism for the marker RM 6100 observed between sterility maintainers and fertility restorers was in accordance with the report of its association with *Rf4* documented by several earlier researchers (Bazrkar *et al*., 2008; Sheeba *et al*., 2009; Bhati *et al*., 2018). The marker RM 216 was also able to discriminate sterility maintainers from fertility restorers by generating allelic variants of 150 and 162 bp in the former, whereas allelic variant of 156 bp in the latter. However, the allelic polymorphism manifesting in the form of smaller differences in the molecular size of the amplified products was not easily distinguishable and unambiguously recognizable.

The studies conducted by several earlier researchers have indicated the additive action of two fertility restorer genes, one (*Rf4*) having larger effect and another (*Rf3*) having smaller effect, in effective fertility restoration of wild abortive type cytoplasmic male sterility system (Sharma *et al*., 2001; Sharma and Singh, 2003; Liu *et al*., 2004; Sharma *et al*., 2005; Ahmadikhah *et al*., 2007; Sattari *et al*., 2008; Cai *et al*., 2013; Chen *et al*., 2017). In the present study, easily recognizable polymorphism between sterility maintainers and fertility restorers was recorded for the markers RM 315 and RM 443, which are known to be linked to *Rf3* gene. Similarly, *Rf4* gene specific polymorphism revealed by the markers RM 258 and RM 6100 located on chromosome 10 was unambiguously observed

between sterility maintainers and fertility restorers. The presence of a major fertility restoration gene *Rf4* in the long arm of this chromosome has been reported by several researchers (Zhang *et al*., 2002; Ahmadikhah and Karlov, 2006; Sattari *et al*., 2007, Sheeba *et al*., 2009; Ngangkham *et al*., 2010; Suresh *et al*., 2012; Cai *et al*., 2013). Therefore, the experimental results led to validation of four microsatellite markers out of eight microsatellite markers used in the present investigation. These four microsatellite primer pairs, namely, RM 258, RM 315, RM 443 and RM 6100 may be further utilized in the identification of effective fertility restorers of wild abortive type cytoplasmic male sterility system in rice.

**Table 1.** List of eight microsatellite primers utilized for targeted amplification of rice genomic regions in fourteen entries under evaluation

| Primer | Ch. No. | Primer sequence (5'-3') | Repeat Motif | An. Tp. ($^0$C) | No. of alleles | No. of shared alleles |
|---|---|---|---|---|---|---|
| RM 171 | 10 | (F) AACGCGAGGACACGTACTTAC (R) ACGAGATACGTACGCCTTTG | $(GATG)_5$ | 52 | 4 | 4 |
| RM 216 | 10 | (F) GCATGGCCGATGGTAAAG (R) TGTATAAAACCACACGGCCA | $(CT)_{18}$ | 48 | 3 | 3 |
| RM 258 | 10 | (F) TGCTGTATGTAGCTCGCACC (R) TGGCCTTTAAAGCTGTCGC | $(GA)_{21}(GGA)_3$ | 56 | 2 | 2 |
| RM 315 | 1 | (F) GAGGTACTTCCTCCGTTTCAC (R) AGTCAGCTCACTGTGCAGTG | $(AT)_4(GT)_{10}$ | 62 | 2 | 2 |
| RM 443 | 1 | (F) GATGGTTTTCATCGGCTACG (R) AGTCCCAGAATGTCGTTTCG | $(GT)_{10}$ | 52 | 2 | 2 |
| RM 1108 | 10 | (F) GCTCGCGAATCAATCCAC (R) CTGGATCCTGGACAGACGAG | $(AG)_{12}$ | 56 | 4 | 4 |
| RM 3873 | 1 | (F) GCTAGCTAGGACCGACATGC (R) CCTCCTCCTTATCCTCCCTG | $(GA)_{50}$ | 58 | 4 | 4 |
| RM 6100 | 10 | (F) TCCTCTACCAGTACCGCACC (R) GCTGGATCACAGATCATTGC | $(CGA)_8$ | 54 | 2 | 2 |

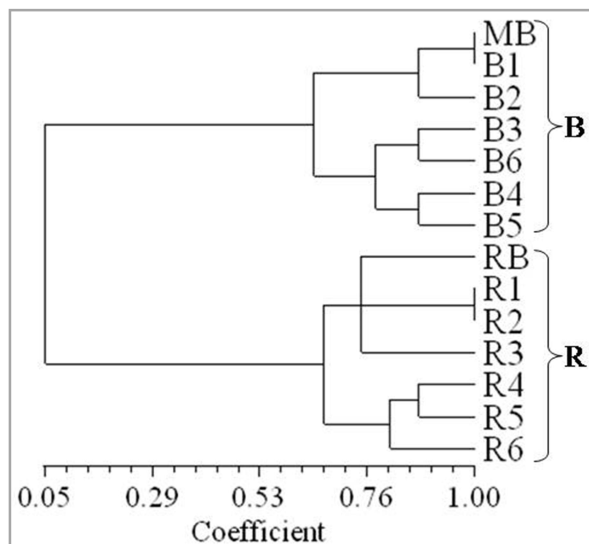


**Fig. 1.** Hierarchical classification based on eight microsatellite markers generated polymorphism between pair-wise combinations of entries.

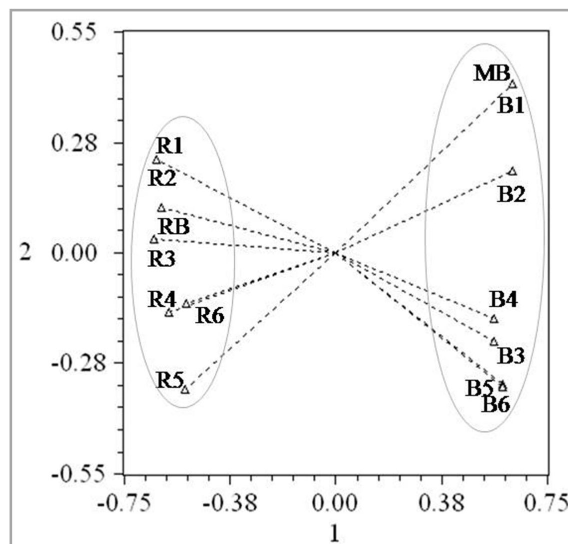


**Fig. 2.** Spatial distribution pattern of eight microsatellite primers based genetic profiles of entries along the two principal axes.

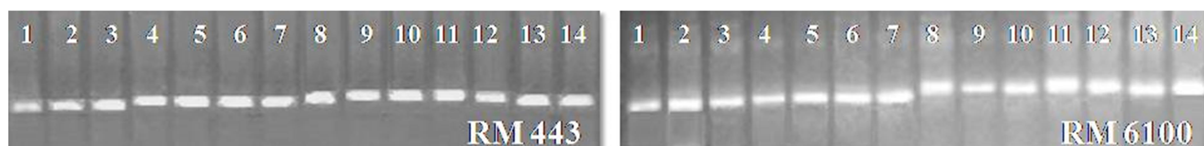


**Fig. 3.** Amplification patterns of targeted genomic regions in fourteen entries (1-MB, 2-B1, 3-B2, 4-B3, 5-B4, 6-B5, 7-B6, 8-RB, 9-R1, 10-R2, 11-R3, 12-R4, 13-R5, 14-R6).

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