

Assessment of Genetic Purity of Parental Lines of Hybrid Rice Using DNA-Based Markers

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Abstract: Assessment of hybrid seed genetic purity is one of the most important quality control parameters in hybrid seed production. With the objective of replacing the GOT with DNA based assays, Cytoplasmic Male Sterile (CMS), restorer and hybrid lines have been screened by means of microsatellite and Sequence Tagged Site (STS) markers. Cytoplasmic Male Sterile (CMS) lines often get contaminated with cognate isonuclear maintainer lines during multiplication. The PCR assay was able to detect precisely the impurities in a commercial seed lot of CMS line. The extent of heterozygosity within parental lines of rice hybrids were assessed and the results suggest that a single, appropriately chosen microsatellite marker should be sufficient for assessing hybrid seed purity.

Keywords: Hybrid Seed, Cytoplasmic Male Sterile, PCR, Heterozygosity

Introduction

Rice, *Oryza sativa* L. is the most important staple crop feeding over half of the world population. The present world population of about 6 billion is likely to cross 8 billion by the year 2030, which necessitates that rice production must increase by 40% to keep pace with the increasing trend in the population growth. Hybrid rice is one of the technological options that will impact the world's rice production (Khush, 2005). Hybrid rice technology appears to be a feasible and readily available option for raising yield potential. In China, about 50% of land under rice cultivation is occupied by hybrid rice, thus making it the largest producer of hybrid rice (Jirong, 2000). Besides, China, hybrid rice is successfully grown in countries like India, Vietnam, Philippines, Latin American countries, to a small extent only. Though the area occupied by hybrid rice in India is still small but it is expected to increase substantially.

Availability of efficient male sterility systems is a prerequisite for the development of hybrids in rice. Mainly, three types of male sterility systems: Genetic Male Sterility (GMS), Cytoplasmic Male Sterility (CMS) and Environment-Sensitive Genetic Male Sterility (EGMS) are well recognized in rice. Of these, the CMS system has been the most effective for hybrid rice breeding. Till date, more than 40 different CMS sources

have been reported in rice (Virmani and Shinjyo, 1988). The discovery of a male-sterility inducing cytoplasm designated „Wild Abortive“ (WA) cytoplasm from a wild rice. The CMS based hybrid breeding system involving male sterile (CMS/A), maintainer (CMS/B) and Restorer (R) lines is popularly known as three line breeding (Virmani, 1993). Currently, two different technologies are adopted for the development of rice hybrids. One is the “three-line system” involving a CMS line, maintainer line and restorer line. The other system is the “two-line system” wherein only two lines (designated as male and female) are involved in the hybrid seed production. Among the CMS lines used for development of three- line rice hybrids, lines based on WA cytoplasm originally derived from a wild rice are the most popular in all countries where hybrid rice is cultivated on a commercial scale (Yuan, 1995).

Essentially, in the CMS lines certain genes are present in the mitochondria, which are responsible for cellular respiration. If these genes are absent or their function is hampered, the mitochondria becomes non-functional as a result of which the viable microspores are not produced as mitochondria follows maternal inheritance Gallagher *et al.*, 2002, Satoh *et al.*, 2004, Yamamoto *et al.*, 2005, Wang *et al.*, 2006). In a self-pollinated crop like rice, one of the challenges is the production and supply of adequate quantities of pure hybrid seed to the farmers. Maintenance of high level of genetic purity of hybrid is essential to exploit the

moderate level of heterosis observed in this crop. The success of hybrid rice technology depends on efficient seed production techniques to ensure timely supply of genetically pure seeds. It has been reported that even 1% impurity in hybrid seed can lead to yield reduction of about 100 kg per ha at farmers' fields (Mao *et al.*, 1996). Indian Seed Act (1966) recommends ensuring 98% genetic purity in commercial hybrid seed lots (Sindhu and Kumar 2002). The required levels of hybrid seed purity can be ensured by using pure parental lines in hybrid seed production. The most commonly observed contamination in hybrid seed production is that of maintainer line getting admixed with CMS line (Yashitola *et al.*, 2004).

For accurate detection of impurities in seed lots, it is essential to identify a set of informative SSR markers which can clearly distinguish the parental lines and amplify specific or unique allele combinations in the hybrids, not present in any other rice line. The fingerprinting of rice hybrids and identification of their genetic relationships are essential for plant improvement, variety registration system, DUS testing, seed genetic purity testing and the Protection of Plant Variety and Breeders "Rights. Accordingly, clear-cut identification of elite crop varieties and hybrids it is essential for protection and prevention of unauthorized commercial use (Nandakumar *et al.*, 2004).

The present work had been undertaken to validate some known markers for discriminating cms lines with their isogenic counterparts, maintainer lines. All the markers employed during the study are PCR-based and are of different nature.

Materials and Methods

Seed Material

Five Cytoplasmic Male Sterile (CMS) lines, five maintainer lines and six restorer lines and two commercial hybrids based on WA cytoplasm were used in the present study (Table 1). The rice germplasms was obtained from Barwale Foundation, Research and Training Centre, Hyderabad. The material has been originally procured from the International Rice Research Institute (IRRI), Philippines.

Seed Germination

Seeds were placed on Petri plates lined with germination paper soaked with water. Petri plates were incubated at $25\pm 2^\circ\text{C}$, for 7-8 days. The papers were wetted with 1 ml water, if required. 10-day-old seedlings were used for DNA extraction (Fig. 1).

DNA Isolation

Genomic DNA was extracted from four-week-old rice seedlings, using modified Dellaporta method (Dellaporta *et al.*, 1983).

Quantification of Genomic DNA

The DNA yield per microgram of seed samples was measured by using a Bio photometer (Bio- Rad) at 260 nm. DNA purity was determined by calculating the absorbance ratio A_{260}/A_{280} . The DNA sample was also quantified on 1% agarose gel electrophoresis.

PCR Amplification

The PCR amplification was carried out in a total volume of 20 μL and the cocktail was composed of 2.5 μL of 10X PCR buffer, 2 μL of 40 mM of dNTP "s mix, 0.5 μL of 25 mM MgCl_2 , 1 μL of 10 μM Primer, 0.5 μL Taq polymerase (3 units μL^{-1}), 11.5 μL of sterile distilled water, 2 μL of genomic DNA (10 ng μL^{-1}). The amplification was carried out in Eppendorf thermocycler. The amplification was carried out at the program comprising of initial denaturation at 94°C for 5 min, followed by 35 cycles of cyclic denaturation at 94°C for 1 min, annealing at 55°C for 1 min, cyclic extension at 72°C for 1 min and finally an extension at 72°C for 5 min. A total of 12 oligonucleotide primers were used for PCR amplification (Table 2). These primers were selected from the literature.

The amplified products were resolved on 1.5% agarose gels in 1X TAE buffer. The size of bands was estimated using a 100 base pair ladder. Bands were visualized under UV light in gel documentation systems and captured images were used for further analyses.

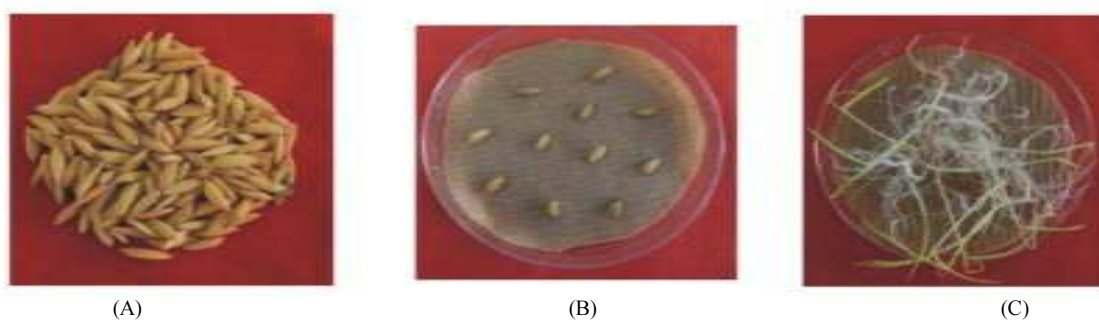


Fig. 1. Seed germination for DNA extraction (A) Seeds (B) Seeds kept for germination (C) 10-day-old seedling

Table 1. Details of rice germplasm used in present study

Sr. no.	Type	Lines	Sr. no.	Type	Lines
1	CMS line	IR 58025 A	2	Maintainer	IR 58025 B
		IR 68888 A			IR 68888 B
		IR 68897 A			IR 68897 B
		IR 79128 A			IR 79128 B
		IR 79156 A			IR 79156 B
3	Restorer	IR 58103-62-3R			
		IR 62037-12-1-2-2-2R			
		IR 65483-14-1-4-13R			
		IR 68445-62-1-3-1R			
		IR 7160-4-1-4-4-4-2-2-2R			
		IR 72R			

Table 2: Details of primers used for amplification during present study

Primer name	Sequence (5'-3')	References
DRRCMS-Forward	5' - ACCTTTGGGCGATGGTT - 3'	(Rajendrakumar <i>et al.</i> , 2007)
DRRCMS-Reverse	5' - GGGTTTAGAGTCGCCAC - 3'	
CMS-Forward	5' - ACTTTTTGTTTT TGTGTAGG - 3'	(Yashitola <i>et al.</i> , 2004)
CMS-Reverse	5' - TGCCATATGTCGCTTAGACTTTAC - 3'	
BFSTS401-Forward	5' - TGCCACTAT TCCACAATGCATG - 3'	(Rajendran <i>et al.</i> , 2007)
BFSTS401-Reverse	5' - CCCTTTCCTGCTTCCCTTTTTTA - 3'	
BFSTS402-Forward	5' - TAGGGCCATGACGGTTTTG - 3'	(Rajendran <i>et al.</i> , 2007)
BFSTS402-Reverse	5' - CGCGTCCTTCCCCAATT - 3'	
pTA248-Forward	5' - AGACGCGGAAGGGTGGTTCCCGGA - 3'	(Yashitola <i>et al.</i> , 2002)
TA248-Reverse	5' - AGACCGGTAATCGAAAAGATGAAAA - 3'	
RMT6-Forward	5' - GATGGTTTTGGAAGGCTG - 3'	(Rajendrakumar <i>et al.</i> , 2007)
RMT6-Reverse	5' - GGGTTTAGAGTCGCCA - 3'	

Results and Discussion

10-day-old seedling was used for DNA extraction. DNA was extracted from all the 18 lines of rice as listed in Table 1. About 10 preparations of each genotype were prepared in order to select the best preparation. Extracted DNA was analyzed using the Bio photometer and the quantity of DNA was assessed on the basis of readings recorded at 260 nm, while the quality of DNA was checked on the basis of ratios of 230, 260 and 280 nm.

PCR amplifications were carried out with six primer pairs (Table 2).

Amplification Pattern Generated by CMS Primer Pair

cms primer pair generated a band of 386 bp in CMS line which was absent in both the maintainer and restorer lines. During the present study, we had analyzed five different CMS lines. As reported in literature four CMS lines viz., IR 58025, IR 68897, IR 79128, IR 79156 generated a band of 386 bp in CMS lines, which was absent in their counter maintainer lines (Fig. 2 A, B and C). Likewise, this band was absent in the entire restorer lines tested (Fig. 2 D). In one CMS line (IR 68888) this band could not be detected even in the single individual genotype analyzed (Fig. 2 B). The lower band present in the gel is below a 100 bp size and it is due to primer dimer so it is not considered during scoring of results. Using this

primer, it was concluded that CMS lines have the band, but it is completely absent in maintainer lines as well as all restorer. However, all the individuals of CMS lines (IR 78128 A and IR 79156 A) show this band uniformly.

Amplification Pattern Generated by DRRCMS Primer Pair

drrcms amplified 130 bp in CMS line and 142 bp in maintainer line. All genotypes of IR 58025 A amplified a single band of 130 bp, while its cognate maintainer (IR 58025 B) amplified two bands one line CMS counterpart i.e., 130 bp and an additional band of 142 bp (Fig. 3 A). In IR 68888 A lines, out of five genotypes tested only in three individuals 130 bp band was detected, while in other two individuals it was absent. In its maintainer line (IR 68888 B), only three individuals developed two bands (130 bp and 142 bp), while in one genotype only 130 bp appeared. In the fifth individual both bands failed to amplify (Fig. 3 B, C). IR 79128 A and IR 79156 A developed a banding pattern similar to the maintainer lines; hence this primer is not suitable for discriminating these two CMS lines from their maintainer (Fig. 3 C). In four restorer lines, four (R1, R2, R3 and R4) both bands (130 bp and 142 bp) developed, however, the intensity of 142 bp band was not very strong. The fifth restorer line (IR 72) did not amplify any of these bands (Fig. 3 D).

Amplification Pattern Generated by RMT 6 Primer Pair

RMT 6 generated multiple bands in both CMS and their cognate maintainer lines. Such banding pattern was almost uniform for all five sets of CMS line pairs (Fig. 4 A-C). However, this primer pair could differentiate the restorer lines from CMS/maintainer lines as it failed to generate any banding pattern in any of the five selected lines (Fig. 4 D).

Amplification Pattern Generated by BF-STS-402 Primer Pair

BF-STS-402 generated a single band of 336 bp in all the lines of tested. This band was uniformly present in all CMS lines along with their maintainers. Furthermore, this band was also present in all restorer lines (Fig. 5 A-D). This primers pair is not suitable for

differentiating any combination of lines used in hybrid rice breeding programs.

Amplification Pattern Generated by BF-STS-401 Primer Pair

BF-STS-401 could differentiate CMS and its cognate maintainer lines. IR 58025 A and IR 58025 B exhibited a differential pattern of discriminating CMS and maintainer lines in comparison to other CMS pairs (Fig. 6 A). In IR 58025 B an additional band (1300 bp) was amplified in addition to a band of 469 bp present in CMS line (IR 58025 A). For other four CMS lines, (IR68888 A, IR 6897 A, IR 79128 A and IR 79156 A) amplified a desired band of 409 bp uniformly, while it was completely absent in respective maintainers for all four cases (Fig. 6 B, C). Likewise, the band was completely absent from all five restorer lines too (Fig. 6 D). Therefore, this primer can be best suited for discriminating maintainers, restorers lines from CMS line.

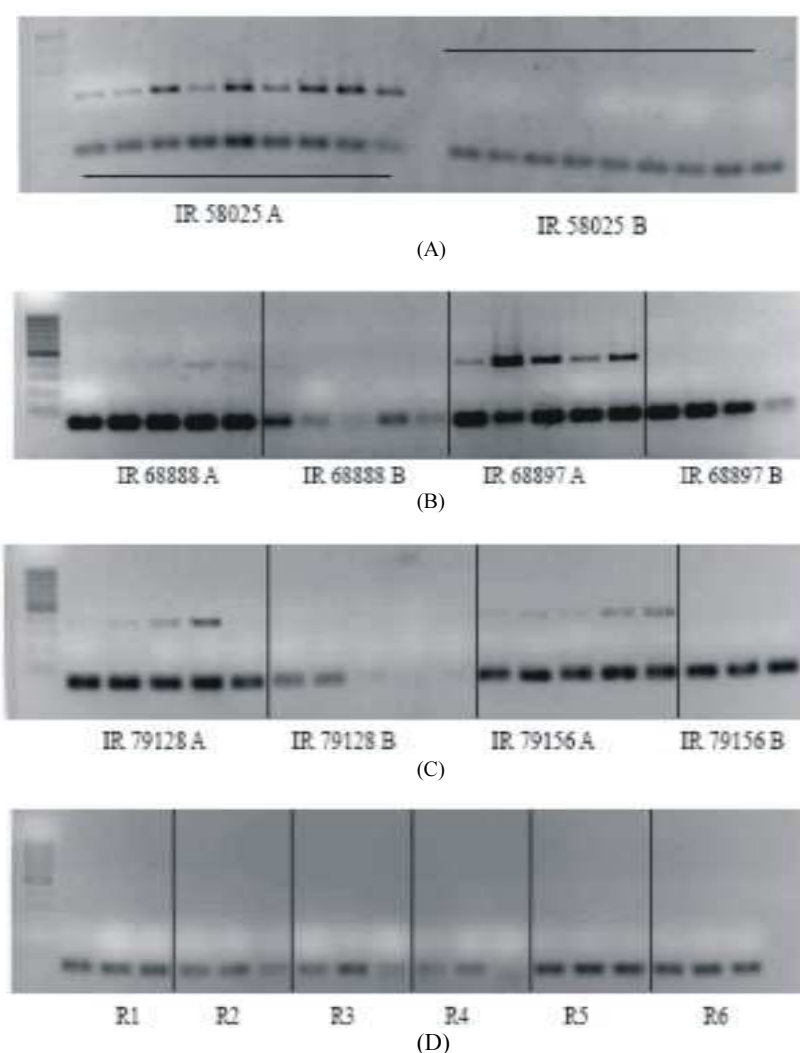


Fig. 2. Amplification profile of rice genotypes: CMS lines and their cognate maintainers, restorer lines, with cms primers

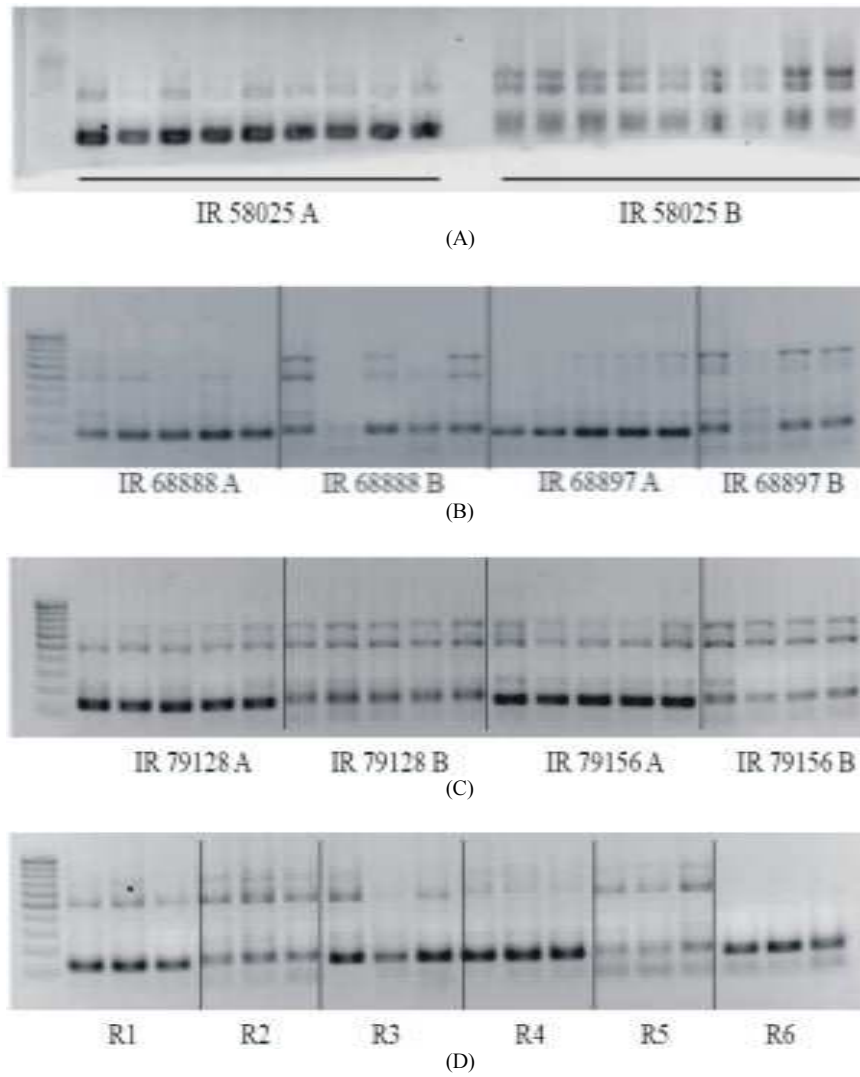
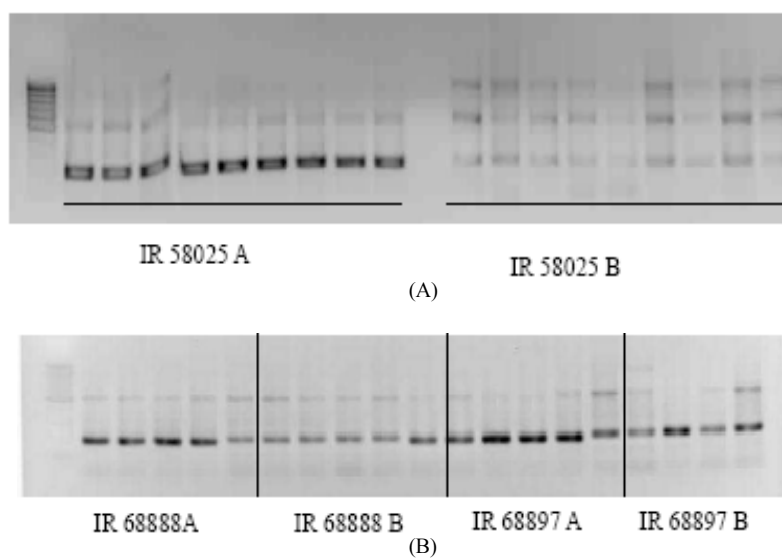


Fig. 3. Amplification profile of rice genotypes: CMS lines and their cognate maintainers, restorer lines, with drcrems primers



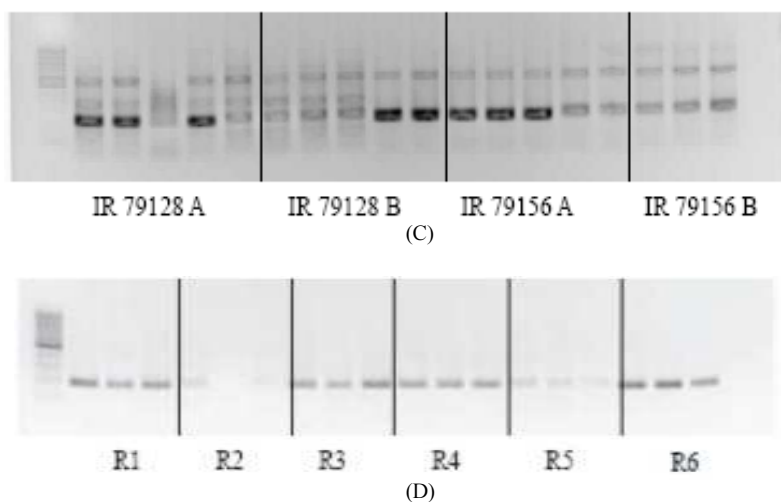


Fig. 4. Amplification profile of rice genotypes: CMS lines and their cognate maintainers, restorer lines, with RMT 6 primers

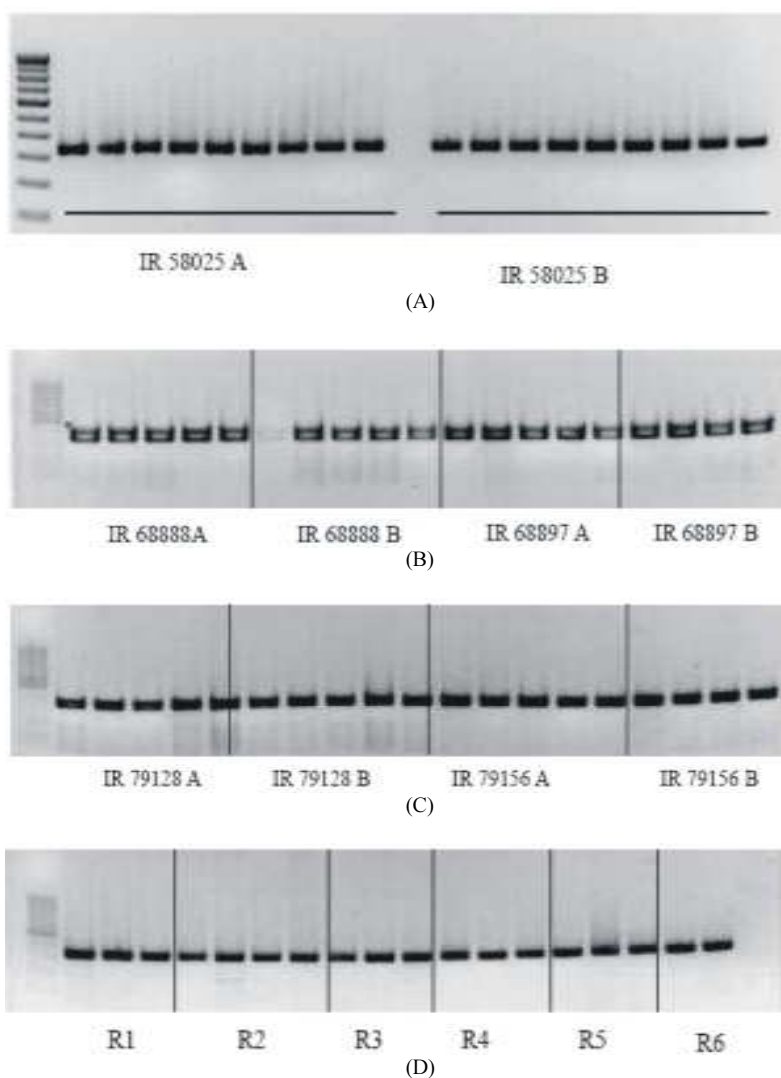


Fig. 5. Amplification profile of rice genotypes: CMS lines and their cognate maintainers, restorer lines, with BF-STS-402 primers

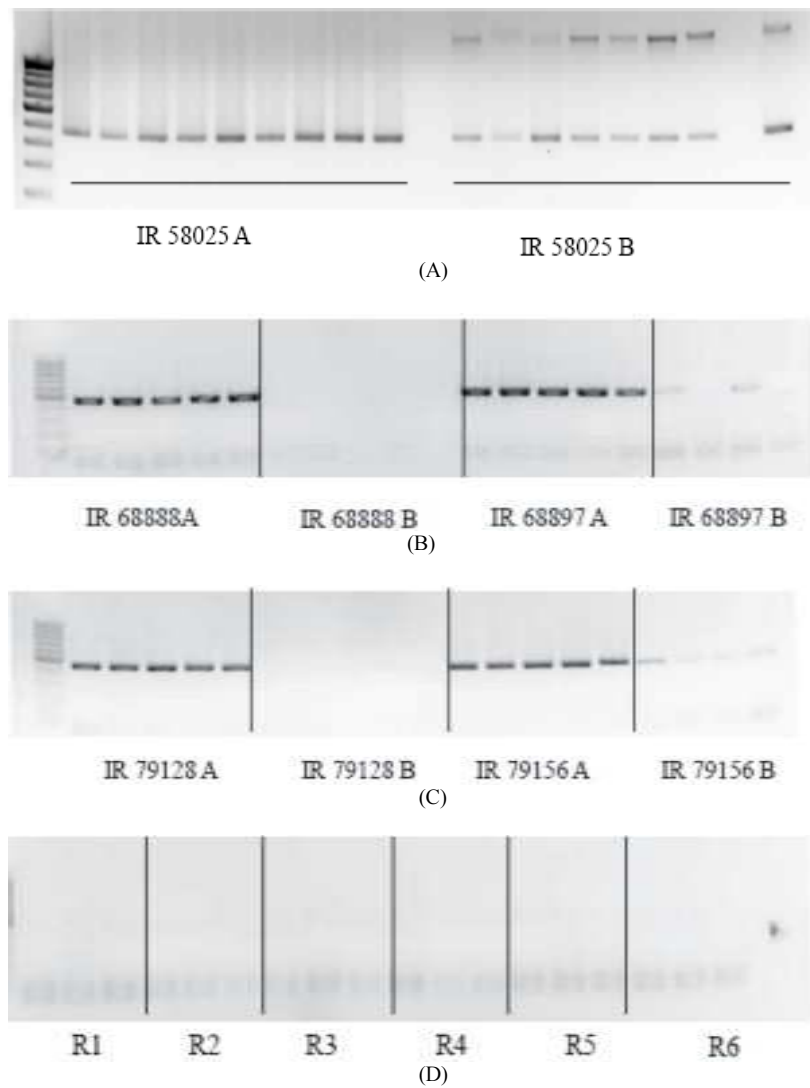
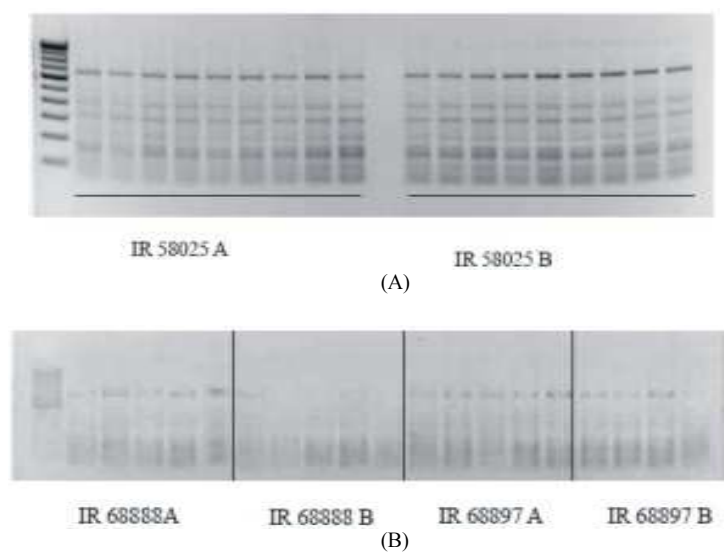


Fig. 6. Amplification profile of rice genotypes: CMS lines and their cognate maintainers, restorer lines, with BF-STS-401 primers



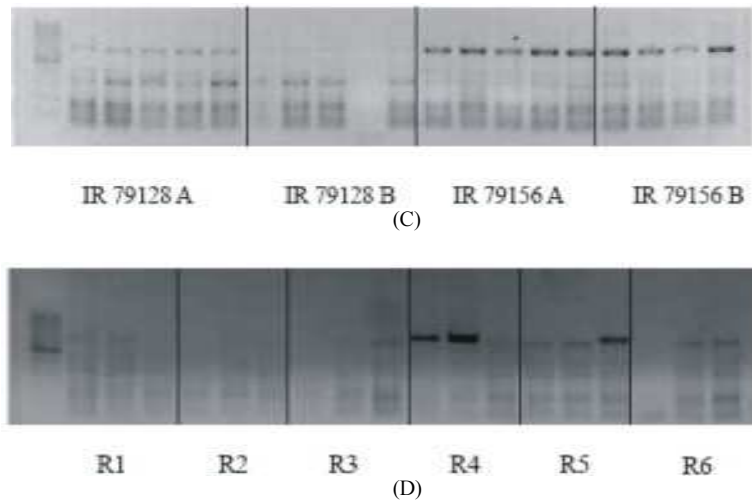


Fig. 7. Amplification profile of rice genotypes: CMS lines and their cognate maintainers, restorer lines, with pTA 248 primers

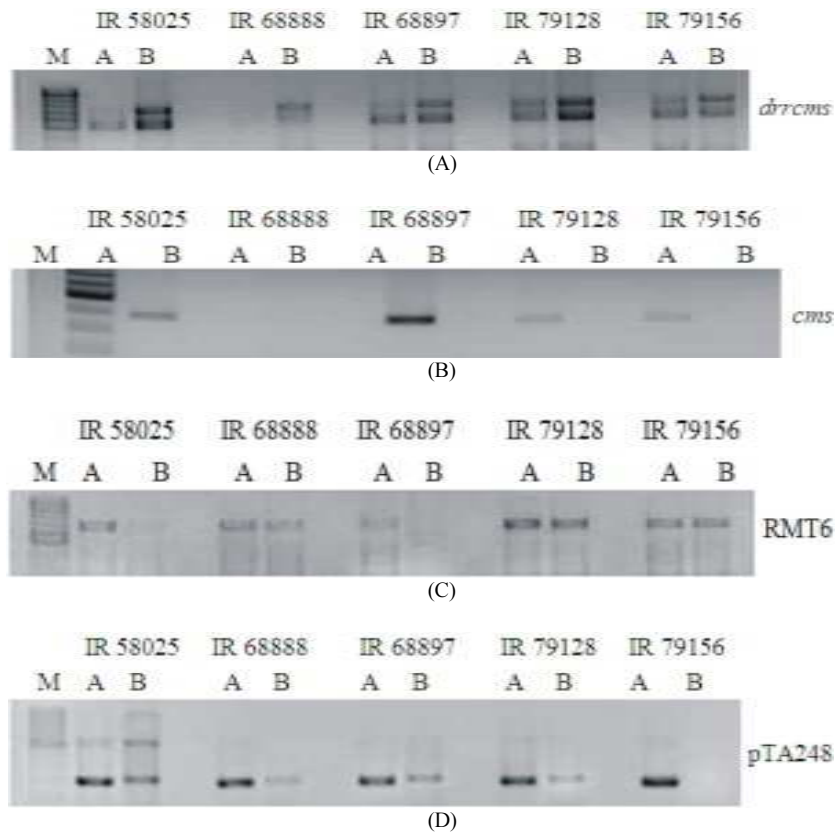
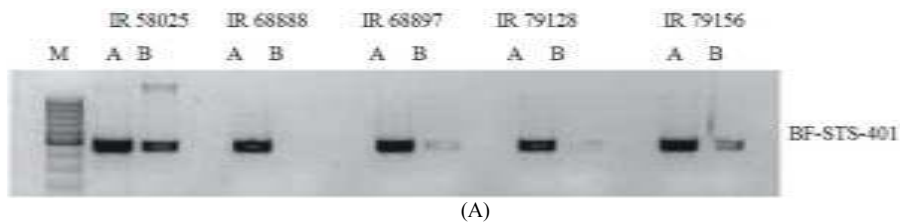


Fig. 8. Validation in five diverse CMS lines having WA sterility along with their maintainers with different known primers



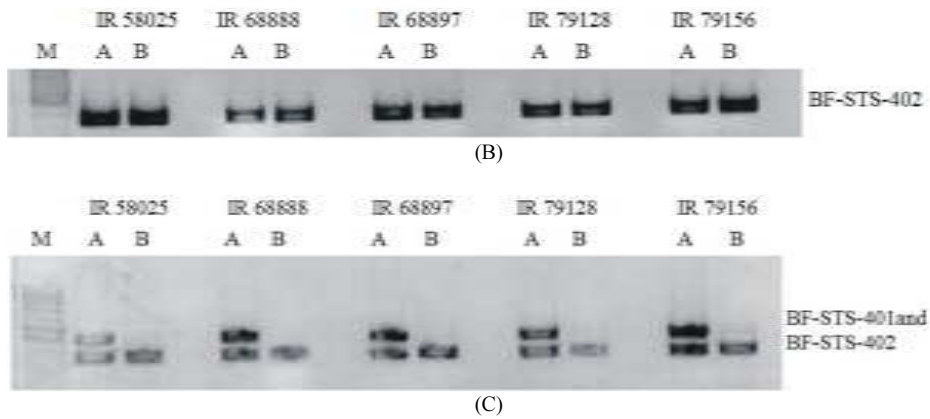


Fig. 9. Validation in five diverse CMS lines having WA sterility along with their maintainers with different known primers

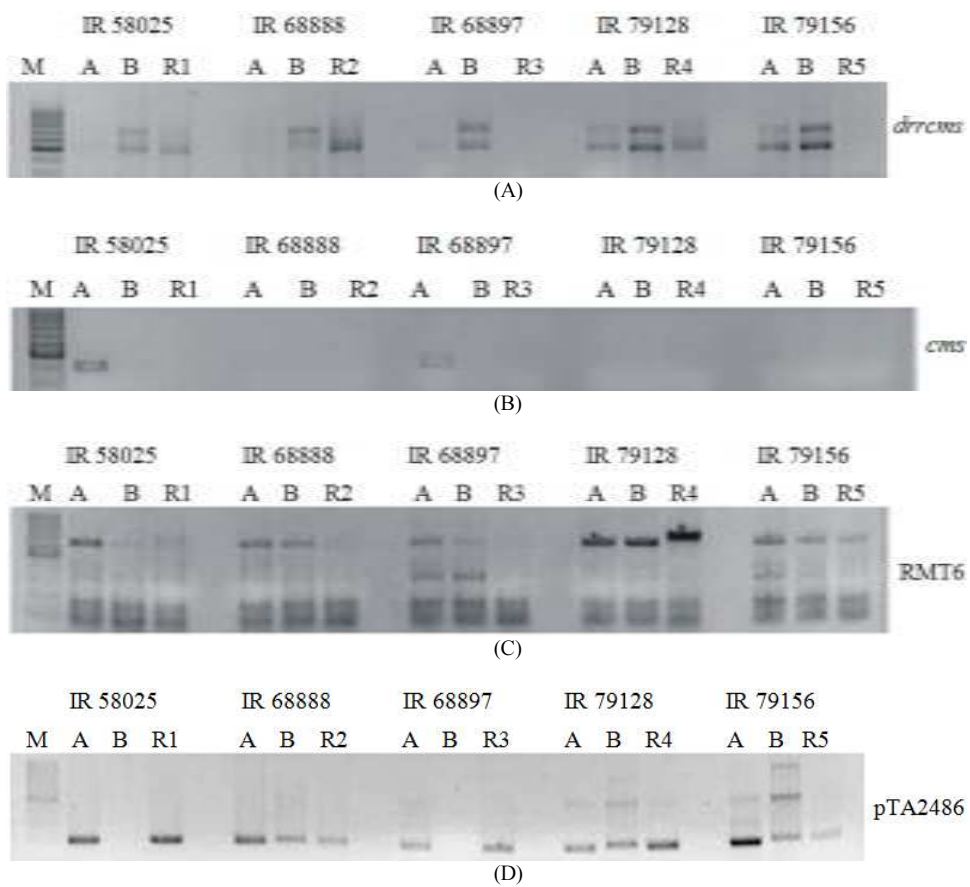
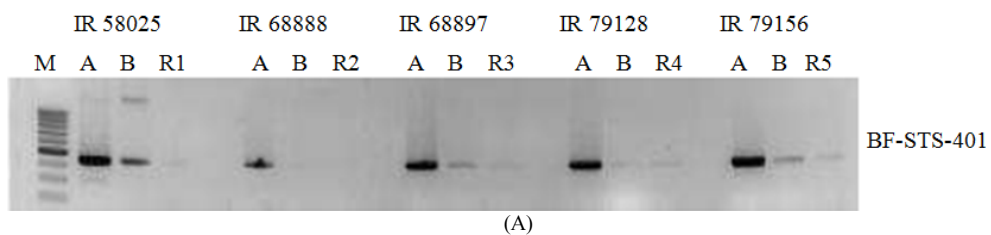


Fig. 10. Validation in five diverse CMS lines having WA sterility along with their maintainers as well as restorers with different known primers



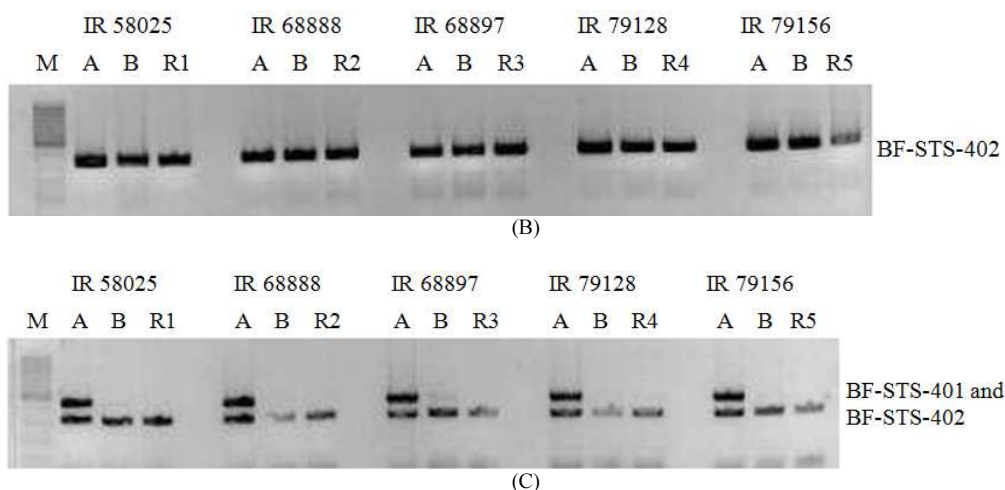


Fig. 11. Validation in five diverse CMS lines having WA sterility along with their maintainers as well as restorers with different known primers

Amplification Pattern Generated by PTA 248 Primer Pair

pTA 248 6 generated multiple bands in both CMS and their cognate maintainer lines. Such banding pattern was almost uniform for all five sets of CMS line pairs (Fig. 7 A-C). Further, this primer pair could not differentiate the restorer lines from CMS/maintainer lines as similar multiple lines were generated in all test lines (Fig. 7 D).

Comparison of Different CMS, Maintainers and Restorer Lines

Comparisons in the banding profile were also studied in CMS lines of 5 different WA cytoplasmic origins for their amplification profile with our tested primers. Additionally, banding profile of all maintainers as well as restorers was also done (Figs. 8-11).

All these five CMS lines have different origins of WA cytoplasm; therefore they have different banding patterns with primers. Only IR 58025 A and B could be differentiated with *drccms* primer. A and B lines IR 68888 did not amplify clear bands. A and B lines of IR 68897, 79128 and 79156 are indistinguishable from each other (Fig. 8 A). Only A lines of IR 58025 and 68897 could amplify bands thereby distinguishing them from their B counterparts by *cms* primer. These bands were completely absent in B lines. A lines of IR 68888, 79128 and 79156 failed to amplify this band (Fig. 8 B). RMT 6 and pTA 248 amplified multiple bands in all lines, thereby making it difficult to differentiate the lines (Fig. 8 C and D).

BF-STS-401 amplified a band of 469 bp in A lines all 5 which was completely absent in B lines of all except that of IR 58025. In this case additional a band of 1300 bp was also present (Fig. 9 A). BF-STS-402 amplified a band of same size uniformly in all lines, thus making it difficult to

discriminate them (Fig. 9 B). However, when BF-STS-401 was multiplex with BF-STS-402, a very clear cut difference was recorded between A and B lines in all 5 cases (Fig. 9 C). When the banding patterns of restorers were combined with A and B lines a very much similar patterns to that of B line were recorded in cases. At most of the times, restorer lines behaved like the B lines (Figs. 10 and 11).

Conclusion

All five CMS lines used in the present study were having the origin of WA cytoplasm. The line IR 25A and its cognate maintainer IR 25 B are the most uniform out of all the other four CMS lines and their maintainers. The amplification profile was comparable for all the primer pairs used for IR 25 A and IR 25 B, as reported in the literature. Most of the commercial hybrids are based on IR 25 A and IR 25 B lines only, the reason behind this could be a high degree of uniformity among individuals to other CMS lines. Primers *drccms*, *cms* and BF-STS-401 could be successful used for analyses of the genetic purity of various lines. Different restorer lines behaved like B lines in their amplification profiles. BF-STS-401 when multiplexed with BF-STS-402 was most suitable for discriminating CMS lines of all 5 origins. A and B lines of IR 58025 and IR 68897 could be identified by *cms* primers, while *drccms* could do so only for IR 58025.

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Author's Contributions

Sunil Bhavsar: Participated in all experiments, Coordinated the data analysis and writing of the manuscript.

Twinkle Solanki: Participated in all experiments and Contributed in writing of the manuscript.

Suchita Amin: Participated in all experiments and Coordinated in data analysis.

Neeru Jain: This work was conceived, planned and co-ordinated.

Ethics

The authors declare that there is no conflict of interests regarding the publication of this paper.

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