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Utilization of SSR Markers for Seed Purity Testing in Popular Rice Hybrids (Oryza sativa L.)

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Abstract: Microsatellite markers were used for fingerprinting of hybrids, assessing variation within parental lines and testing the genetic purity of hybrid seed lot in rice. In this study 35 simple sequence repeats (SSR) markers were employed for fingerprinting 2 popular rice hybrids and their parental lines. Six SSR markers were found polymorphic across the hybrids and produced unique fingerprint for the 2 hybrids. A set of five markers (RM 206, RM 276, RM 204, RM 234 and RM 228) differentiated the 2 hybrids from each other, which can be used as referral markers for unambiguous identification and protection of these hybrids. The analysis of plant-to-plant variation within the parental lines of the hybrid KRH-2 and DRRH-2, using informative markers indicated residual heterozygosity at two marker loci. This highlights the importance of SSR markers in maintaining the genetic purity of the parental lines. To utilize these SSR markers effectively for detection of impurities in hybrids, a two dimensional DNA sampling strategy involving a 20 × 20 grow-out matrix has been designed and used for detection of contaminants in a seed-lot of the popular Hybrids viz., KRH-2 and DRRH-2. The validation of the identified markers was done with conventional grow out test (GOT) and they were comparable with the GOT.

Key words: Hybrid Rice, Molecular Markers, Identification, Genetic purity

INTRODUCTION

Rice is the staple food for a large segment of the Asian population. It has been estimated that rice production in India as well as several other Asian countries must double by the year 2025 to meet the requirements of the increasing population (Hossain, 1996; Paroda, 1998). A self-pollinated crop like rice, one of the challenges is the production and supply of adequate quantities of pure seeds to the farmers. And maintenance of high level genetic purity of hybrid seeds and it is to exploit the moderate level of heterosis in this crop. It is estimated that for every 1per cent impurity in the hybrid seed, the yield reduction is 100 kg per hectare. Thus, there is a need for an assay to assess genetic purity of seeds that is both accurate and faster, so seed produced in the dry season can be released for commercial cultivation in the ensuing wet season.

The genuineness of the variety is one of the most important characteristics of good quality seed. Genetic purity test is done to verify any deviation from genuineness of the variety during its multiplications. Genetic purity test is compulsory for seed certification of all foundation and certified hybrid seeds. Higher genetic purity is an essential prerequisite for the commercialization of any hybrid seeds. Besides, success of any hybrid technology depends on the availability of quality seed supplied in time at reasonable cost. The genetic purity during multiplication stages is prone to contaminate due to the presence of pollen shedders, out crossing with foreign pollens etc., besides physical admixtures. Thus use of seeds with low genetic purity results in segregation of the traits, lower yields and genetic deterioration of varieties.

Traditional GOT based on morphological markers are time consuming and are environmental dependence. To overcome this disadvantage, the molecular markers are being used in many of the crops. However, due to repeatability of the results and accuracy of the obtained results are under question. This made a way for use of molecular markers particularly the co-dominant markers. The SSR markers are of great importance for rapid assessment of hybrid and parental line seed purity (Yashitola et al., 2002, Antonova et al., 2006 and Pallavi et al., 2011).

The primary objective of the present study was to identify the public sector bred Indian two popular rice hybrids, viz., KRH-2 and DRRH-2 together with their parental lines developed recently, using SSR markers, to provide the DNA fingerprint for these rice hybrids and their parental lines, and to establish the basis for identification and monitoring of seed purity for these hybrid rice combinations.

MATERIALS AND METHODS

Plant materials:

For the purpose of molecular identification, 2 public sector rice hybrids viz., KRH-2 and DRRH-2 released for commercial cultivation in different parts of India and their parental lines were selected for this study. The F_1 seeds of KRH-2 and their parental lines IR-58025A (the sterile female), IR-58025B (the maintainer), KMR-3R (the restorer) were obtained from Division of hybrid rice, Zonal Agricultural Research Station, VC Farm, Mandya and DRRH-2 and their seeds of parental lines IR-68897A (the sterile female), IR-68897B

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(the maintainer), DR 714-1-2R (the restorer) were obtained from the concerned breeder, Directorate of Rice Research, Hyderabad. For the purpose of molecular identification seeds of above mentioned hybrids were germinated in aseptic condition grown in greenhouse, NSP, UAS, Bangalore. A random sample of 400 seeds of KRH-2 and DRRH-2 representing the commercial F_1 seed lot was used for testing their genetic purity. Out of 400, 30 randomly drawn F_1 seeds were used for marker analysis and others were used for Grow-out test (GOT). The GOT was conducted at National Seed Project, University of Agricultural Sciences, GKVK, Bangalore during Rabi-2009.

Molecular analysis:

Plant DNA was isolated from seedlings using CTAB (Van der Beek et al., 1999) protocol as follows: about 0.1 g of young leaf tissue for each sample was homogenized in liquid nitrogen, and incubated at 60^0C for 30–45 min with 500 µL of CTAB buffer (1.0M pH 8.0 Tris-HCL, 3ML NaCl, 0.5 EDTA, 1% PVP-360). Then 500µL 24:1 of chloroform: isoamyl alcohol mixture was added and blended thoroughly for 5min. After centrifugation (5 min, 13 000 rpm), aqueous layer was pipetted into a new eppendorf tube and an approximately equal volume of cold ethanol was added. After storage at -20 ^{0}C for 30–60 min, precipitated DNA was centrifuged, vacuum dried and finally stored in TE buffer.

For fingerprinting DNA from the bulk leaf samples of 2 to 5 individual plants was used. Quantification of DNA was accomplished by analyzing the DNA on Nano Photometer analyzer and also on 0.8 % Agarose gel using diluted uncut lamda DNA as standard. DNA was diluted in TE buffer to a concentration of approximately 25ng/µL for PCR analysis. The sequence information for the primer pairs was obtained from the publications of Wu & Tanksley (1993), and Temnykh et al. (2000) from sequence information obtained from DNA libraries and published sequence data (www.gramene.org). A total of 35 hyper variable SSR primer pairs distributed across the 12 chromosomes were used for PCR amplification.

PCR amplification:

Thirty Five SSR primer pairs were selected for this study. PCR was performed in a volume of the reaction mixture was 20 µL containing of 30ng of template DNA, 1 x PCR buffer with 1.5mM of $MgCl_2$, 0.2 mM of each dNTPs, 10 pmol of each primers and 1U of Taq DNA polymerase. PCR was carried out in a Thermal Cycler was used and programmed for 35 cycles of 95^0C (5 min), 94^0C (1 min) 56^0C (30 Sec.), 72^0C (1 min) then followed by final-extension at 72^0C for 5 min. PCR products (10–15 µL) were used for electrophoresis and the amplicons were resolved on 1.5 % agarose gel stained with ethidium bromide at 1 µg/mL, and visualized under UV in a gel documentation system and impurities were identified based on deviations in expected amplification pattern.

Grow-out trials:

The parental and commercial seeds of the two F1 hybrid cultivars were grown as 20 × 20 Grow-out matrix in National Seed project research plots during Rabi-2009 with all the agronomic and plant protection measures were adopted as per recommended package of practices for raising a healthy crop. Genetic purity visual evaluation was conducted based on the main important morphological characters thought the growth period.

RESULTS AND DISCUSSION

Characterization and identification of cultivars are crucial to varietal improvement, release and in seed production programme. It is mandatory to maintain the genetic purity of hybrid seed for the successful crop production. Unambiguous characteristic pattern of hybrids can be obtained using DNA markers and had been termed as DNA fingerprinting. The use of DNA markers to obtain genotype specific profiles had distinct advantages over morphological and biochemical methods. The morphological markers are influenced by the environmental conditions, labour intensive and time consuming. However, the biochemical markers such as isozyme and protein patterns are least influenced by the environment but exhibit limited polymorphism and often do not allow discrimination between closely related inbred lines (Lucchese et al., 1999). DNA markers overcome most of these disadvantages of morphological and biochemical markers that can be useful to distinguish varieties and off types. The usefulness of DNA fingerprinting technique for cultivar identification was demonstrated by Dallas (1988) for the first time in rice.

The present study utilized the SSR marker techniques for identification of two Rice hybrids along with their parental lines, demonstrating that this technique can be successfully applied to distinguish and identify the hybrids from its parental lines. SSR had much more polymorphism than most of other DNA markers, and is co-dominant and large in quantity. Therefore, the high polymorphic information content (PIC) of SSR had promoted the application of microsatellites as molecular markers in fingerprinting (Ashikawa et al., 1999).

In this study primer pairs of 35 SSR's primer pairs associated with each hybrid and parental lines were assessed on 1.5 to 2.00 per cent agarose. The PCR products of the DNA samples on the agarose did appeared and showed polymorphism among the hybrids and their parental lines.

Among the 35 primers studied, ten primers viz., RM 234, RM 206, RM 276, RM 219, RM 216, RM 209, RM 204, RM 228, and RM 335 showed polymorphism between the parental lines which were used for the production of rice hybrids KRH-

2and DRRH-2 and rest of primers showed monomorphic banding pattern (Fig. 1). The male sterile line IR-58025A of hybrid KRH-2 and its restore line KMR-3R amplified an allele of size 120bp-140bp, while DRRH-2 CMS line IR-68897A and its restorer line DR 714-1-2RR did amplified the allele of size 100 to 210bp.

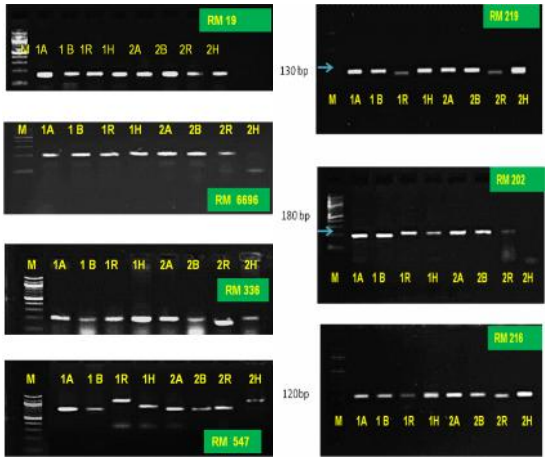

Figure.1: SSR markers profile showing the monomorphic banding pattern of rice hybrids and their parental lines. Lane 1A: IR-58025A, Lane 1B: IR-58025B, Lane 1R: KMR-3R, Lane 1H: KRH-2, lane 2A: IR68897A, Lane 2B:IR68897B, Lane 2R: DR 714-1-2R, Lane 2H: DRRH-2

The two rice hybrids studied viz., KRH-2 and DRRH-2 were able to distinguish from their parental lines using a specific SSR marker. Based on the complementary banding patterns between the hybrids and their parents the SSR marker RM 206, RM 234 and RM 276 were identified as the three specific markers to distinguish F_1 hybrid KRH-2 form their parental lines (fig.2 & 3).

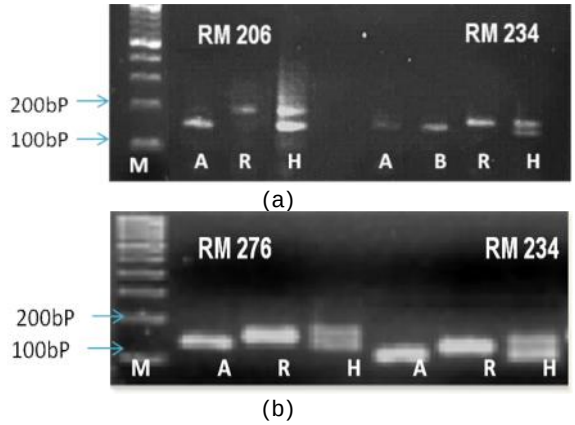

Figure 2: Polymorphic SSR markers profiles confirming hybridity of KRH-2 obtained with (a) RM 206 and RM 234 (b) RM 276 and RM 234. M=Marker 100bp ladder,
A=IR-58025A,
B= IR-58025B, R= KMR-3R, H= KRH-2.

The RM 206 amplified a specific allele of size 130 bp in F_1 KRH-2; seed parent IR-58025A and in its maintainer line but not in pollen parent (KMR-3R). While the allele size of 120 bp was absent in pollen parent. The same SSR marker RM 206 had amplified allele of size 130bp in restorer parent (KMR-3R) which had restored the fertility in male sterile parent. The same allele size of 130 bp has also appered in F_1 hybrid but not in female parent (IR-58025A). Thus, it confirmed that the allele of size 130 bp is very specific to the pollen parent.

Thus, presence of both female and male parent alleles was observed as a resultant of crossing between two parents (F_1 hybrid). This confirmed the crossing and hybridity between two parents. The appeared banding pattern is highly specific to the KRH-2 and not observed DRRH-2 hybrid. Similarly RM 234 maker had also resulted in amplifying allele of size 120 bp in female parent (IR-58025A) and maintainer line (IR-58025B), which was absent in pollen parent (KMR-3R). While, the pollen parent had an amplicon at 130bp which was absent in female parent (IR-58025A). However, the F_1 hybrid exhibited both the alleles of the parents confirming the heterozygosity condition of the hybrid by having bands at 120 and 130bp. The identified SSRs in F_1 hybrids showed complementary banding pattern of both parents. It was valuable to distinguish the F_1 from their male and female parents.

Similarly, the newly released hybrid rice DRRH-2, could be identified and distinguished by the SSR marker RM 204, RM 234 and RM 228. The marker RM 234 had amplicon of size 120bp size in its female parent (IR-68897A) and its maintainer line (fig. 3). The same marker had another amplicon of size 130bp in pollen parent (DR 714-1-2R).

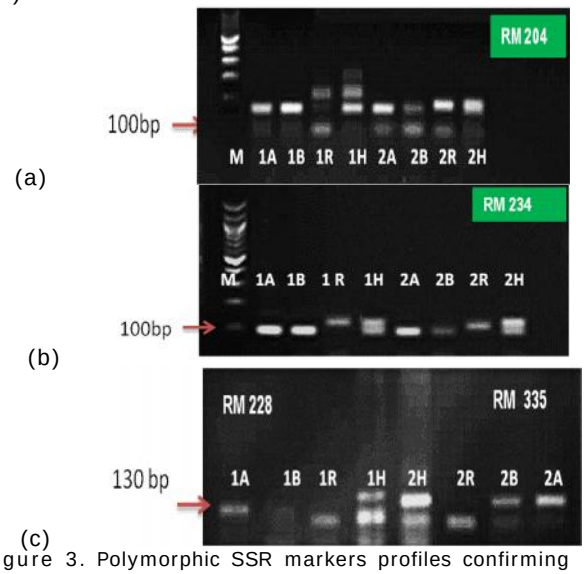

Figure 3. Polymorphic SSR markers profiles confirming hybridity of DRRH-2 obtained with (a) RM 234 (b) RM 204 (c) RM 228 and RM 335. M=Marker 100bp ladder, 1A=IR-68897A, 1B= IR-68897B, 1R= DR 714-1-2R, 1H= DRRH-2, 2A=IR-58025A, 2B= IR-58025B, 2R= KMR-3R, 2H= KRH-2.

The banding pattern of this hybrid showed both the amplicon at 120bp and 130bp. Thus it is

confirmed the genuine crossing and heterozygotic condition of the hybrid. The SSR markers identified had both female and male specific bands and are useful in genetic purity testing. These markers have an advantage of co-dominance inheritance, easy scoring of the alleles, reproducibility and accessibility to laboratories (Paniego et al., 2002). The use of SSR markers for genetic purity testing has been demonstrated in maize (Wang et al., 2002); in rice (Nandakumar et al., 2004); in sunflower (Pallavi et al., 2011).

A cytoplasmic male-sterile (CMS) system is desirable for use in hybrid seed production as it eliminates the need for hand emasculation. CMS is a maternally inherited plant trait characterized by the inability of flowers to produce viable pollen but without affecting the female fertility and it is often associated with mitochondrial DNA rearrangement, mutation, and editing. CMS lines were multiplied with adequate isolation distance leaving no scope for a biological contamination through foreign pollens coming from nearby Rice fields. Under such circumstances, the only impurity that can be expected in CMS line seed lot that comes from its maintainer line probably could be as mechanical admixture during various stages of seed handling of CMS lines.

An attempt was made to distinguish CMS lines (male sterile) used for the hybrid seed production from its maintainer line (male fertile) using SSR markers. The DNA samples of CMS lines and its maintainer lines were amplified with primer pairs and resolved in agarose gel (2 %). The primers pairs studied did not show good polymorphism between A lines and B lines. However, the banding pattern could not able to distinguish CMS A line from its maintainer CMS B line. In Rice the cytoplasmic male sterility is caused by a mutation in the mitochondrial genome (mtDNA) and male sterile and male fertile Rice lines differ in a 17 kbp fragment only (Korell et al., 1992). This had resulted in isogenic lines and differs only in one gene for pollen fertility. The gene responsible for pollen fertility is present in the mitochondrial genome and need to isolate for distinguishing male fertile form male sterile line (Begona et al., 2005).

Field performance of the rice hybrid individuals:

In the Grow-out trials, purity evaluation was conducted based on morphological traits including plant height and days to maturity, pollen sterility, and presence of panicle awns, panicle exertion, panicle length, nodal pigmentation and flag leaf senescence. The characters of few individuals shown deviation from the standard characters were identified as off-type and they were similar to those of the male parental type, which was also supported by the molecular marker testing.

The results of the field grow-out test (GOT) and SSR marker test were comparable. For comparison of GOT test results with molecular GOT, leaf sample collected by random sampling were evaluated by using primer 'RM 206' for KRH-2 hybrids and RM 234 for DRRH-2 hybrids revealed 100, 93.34 and 91.43 per cent of off-types which were validated with the theoretical purity (Field GOT) per cent of 100, 95 and 90 per cent, respectively. In this study, the validation of the identified markers was done with conventional grow out test (GOT) and they were comparable with the GOT.

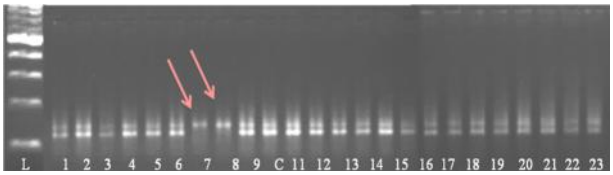

Figure.4: Genetic purity testing of KRH-2 hybrid seeds using the SSR marker RM 206 L= 100 bp ladder, Lane 1-23 = individual F1 plants representing a random sample from hybrid seed lot of KRH-2. Lane 7 and 8 (arrow) represents an off-type, a contaminant.

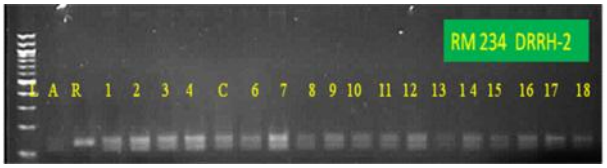

Figure.5: Genetic purity testing of DRRH-2 hybrid seeds using the SSR marker RM 234; L= 100 bp ladder, Lane 1-18 = individual F1 plants representing a random sample from hybrid seed lot of DRRH-2.

The present study showed that SSR markers are quick, effective and results are generally consistent with morphological analysis in the field study. Primers identified in the study could be utilized for routine genetic purity testing of KRH-2 and DRRH-2 hybrids. The SSR marker information developed through this study will be of immense help for hybrid rice seed industry to select appropriate marker combinations and assess genetic purity of the crop.

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