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Identification of SSR markers for hybrid purity testing in newly released rice hybrid KRH-4

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Abstract

Microsatellite markers were used for fingerprinting of hybrid, assessing variation within parental lines and testing the hybrid purity of hybrid seed rice. In this study 25 simple sequence repeats (SSR) markers were employed for fingerprinting for newly release rice hybrid (KRH- 4) and their parental lines. Polymorphic primers viz., RM202, RM204, RM219, RM216, RM1385, RM21, RM336, RM209, RM7279 and RM206 could clearly distinguish KRH-4 from its parental lines. The polymorphic primer RM216 amplified an allele size of 160bp in CRMS32A (A) and 150bp in MSN36 (R). Similarly RM204 amplified an allele size of 110bp in CRMS-32A (A) and 120bp in MSN36 (R). RM7279 amplified an allele size of 190bp in CRMS32A (A) and 1800bp in MSN36 (R). Since the bands of RM204, RM216, and RM7279 of KRH-4 hybrid rice were complement type of their parents, clear and distinct that can be utilized for purity assessment of hybrid rice KRH-4.

Highlights

- SSR marker technique was utilized for characterization and identification of CRMS32A (A), MSN36 (R) and KRH-4 (H) demonstrating that this technique can be successfully applied to distinguish and identify the hybrid from its parental lines.

Keywords: Rice hybrid, quantification, SSR marker, identification

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. 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 1% impurity in the hybrid seed, the yield reduction is 100 kg per hectare. (Mao *et al.* 1992) 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 demerit, 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 away 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, Chethan kumar *et al*. 2012 and Pallavi *et al*. 2011).

The primary objective of the present study was to identify the public sector bred Indian popular rice hybrid, *viz.,* KRH-4 with their parental lines developed recently, using SSR markers, to provide the DNA fingerprint for these rice hybrid and their parental lines, and to establish the basis for identification and monitoring of seed purity for these hybrid rice combinations.

Materials and Methods

For the purpose of molecular identification of newly released rice hybrid *viz.,* KRH-4 released for commercial cultivation in different parts of India and their parental lines were selected for this study. The F_1 seeds of KRH-4 and their parental lines CRMS-32A (the sterile female) and MSN-36 (the restorer) Seeds of parental lines and hybrid were grown in nursery beds at Zonal Agricultural Research Station, VC, Farm, Mandya. Fully expanded healthy leaves were collected from 20 days old seedlings, rinsed with water and surface sterilized with alcohol (70%) and used for the extraction of DNA.

Molecular Analysis

Genomic DNA extraction

Total genomic DNA was extracted from leaves from the hybrid and its parental lines using CTAB method as outlined by Ven der Beek *et al.* (1999) with slight modifications. The reagents and the buffer solutions used for DNA extraction.

Sample preparation

Seeds of parental lines and hybrid were grown in nursery beds at Zonal Agricultural Research Station, VC, Farm, Mandya. Fully expanded healthy leaves were collected from 20 days old seedlings, rinsed with water and surface sterilized with alcohol (70%) and used for the extraction of DNA.

Extraction Protocol

- About five grams of leaf tissue was homogenized in a tissue grinder by adding pinch of PVP, 20 ml of extraction buffer (Appendix IV) and a bead.
- The contents were transferred to centrifuge tubes and incubated at 65^0C for 30 min.
- By cooling it to room temperature, equal volume of wet chloroform was added and mixed gently. The mixture was then centrifuged at 4000 rpm for 15 minutes at room temperature.
- The aqueous solution was pipetted out into new centrifuge tube and again equal volume of wet chloroform was added and mixed gently.
- Finally, the samples were centrifuged at 4000 rpm for 10 minutes and the supernatant was tipped out into another new tube and double volume of chilled isopropanol was added and refrigerated overnight at -20°C.
- There was precipitation of suspended DNA in the sample. Centrifuged at 8000 rpm for 10 minutes.
- Ethanol was discarded and pellets were air dried by inverting the tubes over night. The pellets were re-suspended in TE buffer and stored at -80 ^{0}C for further use.
- Supernatant was discarded and 100 µl of ethanol (70%) was added to wash the pellet once or twice and air dried the pellet.
- The pellet is dissolved in 20 µl of TE buffer and store at -20°C until further use.

Quantification of DNA by agarose gel electrophoresis

- The gel casting plate and the comb was sterilized using 70% ethanol placed properly

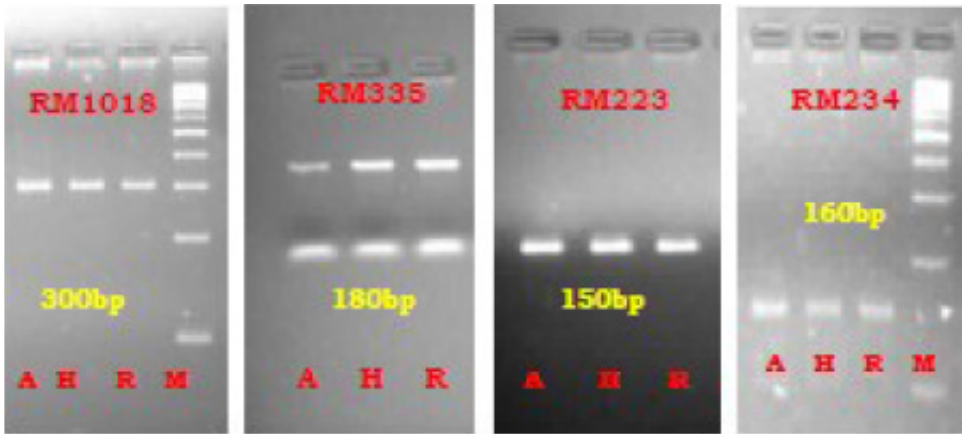

Fig. 1: Monoymorphic banding pattern of rice parental lines and hybrid using SSR marker

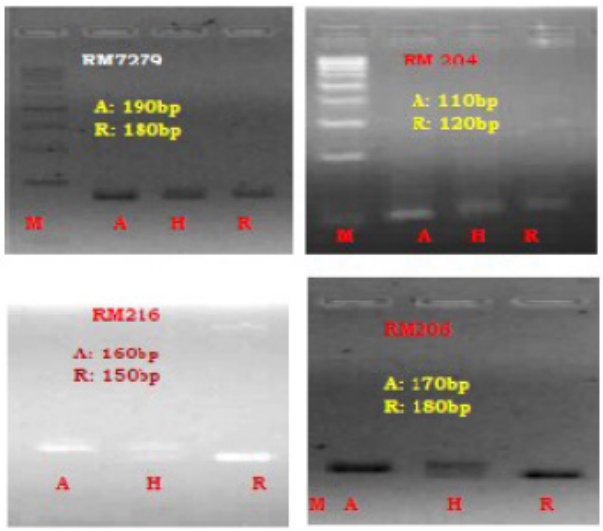

Fig. 2: Polymorphic banding pattern of rice parental lines and hybrid using SSR marker.

in casting plate kept on a perfectly horizontal platform.

- The DNA was quantified by electrophoresis in 0.8 % Agarose gel using 1xTBE electrode buffer (8g in 100ml) and Ethdium bromide (50ng/ml of Agarose) as staining chemical. Agarose was dissolved by heating in microwave oven and then allowed to cool for few minutes until the temperature reaches to 55-60^{0}C. To this ethidium bromide (0.5µg/ml) was added and poured into the gel mould and allowed to solidify.
- The combs were removed carefully after solidification of the agarose.
- The casted gel was placed in the electrophoresis unit with wells towards the cathode and submerged with 1x TBE buffer to a depth of about 1cm.
- About 2 µl of DNA sample from hybrid and its parental lines was mixed with 2 µl of loading dye (50 % sucrose + 5 µl of bromophenol blue and xylene cyanone) and loaded in to the gel.
- Known quantity of 'λ' uncut DNA (100ng and 200ng) was also loaded as reference to quantify the DNA.
- The electrophoresis was carried out at 80 Volts for one hour.
- The gel was viewed and documented using a gel documentation system to ascertain the quantity of DNA isolated in samples.

SSR Analysis

Microsatellites can be easily amplified by the Polymerase Chain Reaction using sequences which are complementary to flanking sequence as

forward and reverse primers. These forward and reverse primers have been developed using the unique sequences that flank microsatellites (Weber and May, 1989). The sequence information for the primer pairs was obtained from the publications of 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. In general, primer pairs have been derived from sequence information obtained from DNA libraries and published sequence data (www.gramene.org). A total of 25 hyper variable SSR primer pairs distributed across the 12 chromosomes were used for PCR amplification.

Microsatellite analysis was done with the following steps:

1. PCR amplification of genomic DNA was done using forward and reverse microsatellite primers (Primers were obtained from Sigma Aldrich).
2. Resolution of polymorphism through agarose gel electrophoresis.
3. Analysis of banding pattern.

PCR amplification

Twenty Five SSR primer pairs were selected for this study.PCR was performed in a volume of the reaction mixture was 15 µL containing of 30ng of template DNA, 1 x PCR buffer with 0.3 mM of $MgCl_2$,1.0 mM of each dNTPs, 1.0pmol 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 95^0 C (5 min), 94^0 C (30 seconds), 58^0C (30 Sec.), 72^0C (1 min) then followed by final-extension at 72^0C for 5 min. PCR products(15 µL) were used for electrophoresis and the amplicons were resolved on 3.0 % agarose gel stained with ethidium bromide a 1.0µg/mL, and visualized under UV in a gel documentation system and impurities were identified based on deviations in expected amplification pattern.

Results and Discussion

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. 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 *et al*. (1988) for the first time in rice.

The present study utilized the SSR marker techniques for identification of Rice hybrid along with their parental lines, demonstrating that this technique can be successfully applied to distinguish and identify the hybrid 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 25 SSR'sprimer pairs associated with each hybrid and parental lines were assessed on 3.00% agarose. The PCR products of the DNA samples on the agarose did appeared and showed polymorphism among the hybrid and their parental lines.

The present study, the hybrid KRH-4 (H) and its parental lines CRMS32A (A), MSN36 (R) were characterized using 24 SSR primers distributed uniformly across the chromosome. As expected only one allele was detected in a hybrid when the parents were monomorphic for a particular microsatellite locus and two alleles (one allele per parent) were present in hybrid when polymorphism was detected between the CMS and restorer lines. Among the twenty five SSR primers studied, all of them generated PCR products of unusual

base pairs. The results of all amplified products based on the banding pattern from the present study are presented in and Among the used SSR primers, 15 primers *viz.*, RM206, RM276, RM202, RM204, RM263, RM216, RM219, RM6844, RM1385, RM228, RM6696, RM21 RM209, RM7279 and RM336 exhibited a complementary banding pattern between hybrid KRH-4 and its parental lines with different amplicons as represented in whereas rest of the 11 primers (RM1018, RM223, RM258, RM234, RM164, RM335) were found to be monomorphic primers.

Among the polymorphic primers, RM202, RM204, RM219, RM216, RM1385, RM21, RM336, RM209, RM7279 and RM206 could clearly distinguish KRH-4 from its parental lines and parental lines from one another. The polymorphic primer RM216 amplified an allele size of 160bp in CRMS32A (A) and 150bp in MSN36 (R). Similarly RM204 amplified an allele size of 110bp in CRMS-32A (A) and 120bp in MSN36 (R). RM7279 amplified an allele size of 190bp in CRMS32A (A) and 1800bp in MSN36 (R). Since the bands of RM204, RM216, and RM 7279 , KRH-4 hybrid rice were complement type of their parents, clear and distinct that can be utilized for purity assessment of hybrid rice KRH-4. Thus an informative SSR marker RM216 can be effectively used to differentiate the parental lines from hybrid.

Assessment of seed purity is one of the most important quality control components in hybrid seed production. Genetic purity can be assessed by many methods but each method as various limitations. 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. Traditionally, it has been the practice to carry out a grow out test, based on morphological traits, for assessment of purity of seeds but it is time consuming, space demanding, and often does not allow the unequivocal identification of genotype. The usefulness of DNA fingerprinting technique for cultivar identification was demonstrated by Dallas (1988) for the first time in rice. The utility of SSR markers for finger printing of rice hybrid has been reported by Nandakumar *et al*. (2004).

In the present study an SSR marker technique was utilized for characterization and identification of CRMS32A (A), MSN36 (R) and KRH-4 (H) demonstrating that this technique can be successfully applied to distinguish and identify the hybrid from its parental lines. SSR markers are highly polymorphic than most of the other DNA markers, and are co-dominant in nature. Therefore, the high polymorphic information content (PIC) of SSR had promoted the application of microsatellites as molecular markers in fingerprinting (Ashikawa *et al*. 1999) of crop varieties. In this study, among the tested 24 SSR primers, RM206, RM204, RM202, RM276, RM263, RM216, RM219, RM6844, RM1385, RM228, RM6696, RM21, RM209, RM7279, RM336 (Fig. 2) exhibited a polymorphism between CRMS32A (A), and MSN36 (R) amplifying a PCR product of different base pair. While RM1018, RM223, RM547, RM335, RM258, RM223, RM247, RM164, RM234, RM19 (Fig. 1) were monomorphic primers. Polymorphic primers revealed an amplicon of different base pair ranging from 90bp to 210bp in CRMS32A (A) whereas 100bp to 200bp in MSN36 (R). Monomorphic primers revealed an amplicon ranging from 140bp to 300bp. Among the polymorphic primers, RM216, RM204, RM7279 and RM206 exhibited a clear differential banding pattern between CRMS32A (A) and MSN36 (R) with an amplicon of different base pair. Further RM216 can be used for purity assessment of hybrid (KRH-4).

The present study showed that SSR markers are quick, effective and results are generally consistent with hybrid purity study. Primers identified in the study could be utilized for routine genetic purity testing of KRH-4 hybrid. 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 hybrid purity of the crop.

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