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Identification and Validation of SSR Markers for Genetic Purity Testing in Hybrid Rice

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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 simple sequence repeats (SSR) markers were employed for fingerprinting two popular rice hybrids and their parental lines. The primers RM21, RM1385, RM 444 and RM 346 identified as unique markers for KRH-4 whereas, RM 206, RM 263, RM 164 and RM 276 for hybrid rice KRH-2 and also they were unique compared to SSR markers identified for other public bred hybrids to determine the genetic purity of seed lots and these markers can be used as referral markers for unambiguous identification and protection of these hybrids.. These SSR markers showed that both alleles of the parental lines in pure hybrids proved the heterozygosity, could also identify the off-types and the selfed seeds from its respective F_1 hybrid seeds. The study suggested that the high discriminating power and reliable PCR assays based SSR markers can be used as an effective tool in hybrid seed purity analysis since this technique is simple to use, more accurate and not affected by environment as compared to conventional grow out test.

Keywords : Genetic purity, grow-out test, hybrid rice, SSR polymorphic markers

Rice (*Oryza sativa* L.) is a staple food crop of the world and a principal food crop after wheat in India. Hybrid rice cultivation offers an opportunity to increase yield potential and ensure supply of rice (Virmani and Ish Kumar, 2004). Although, the hybrids are costly, the farmers grow hybrids because of higher seed yield and in turn higher economic returns, which necessitates the production of fresh seed every year. It is estimated that for every one per cent impurity in the hybrid seed, the yield reduction is 100 kg per hectare (Mao *et al*., 1996). Hence, the assessment of seed purity is one of the most integral part of quality control components in hybrid seed production. Conventionally, the genetic purity of the rice hybrids were assessed by the Grow Out Test (GOT) that involves growing plants to maturity and assessing several morphological and floral characteristics that distinguish the hybrid. But the sensitivity of morphological traits to the environment, further arrest the application of GOT for genetic purity assessment. The utility of these SSR markers in identification of rice hybrids, their respective parents for assessment of plant to plant variation within the parents and testing the genetic purity of rice hybrids has been supported earlier in the commercial hybrids (Sundaram *et al.,* 2008; Chethan kumar *et al.,* 2012 and Nethra *et al.,* 2016). The present study was formulated in order to identify a set of informative SSR markers for rice hybrids (KRH 2 and 4) and to validate these unique informative markers for genetic purity testing of rice hybrids.

Material and Methods

Rice hybrids (KRH-2 and KRH-4) and their parental lines were obtained from Zonal Agricultural Research Station, V.C. Farm, Mandya. For the purpose of marker analysis, seeds of parental lines and hybrids were germinated in pots under poly house conditions at the National Seed Project (Crops), GKVK, University of Agricultural Sciences, Bengaluru during 2016-17 in order to identify and validate the unique markers for KRH-2 and KRH-4 hybrids. However, to validate the informative SSR markers for genetic purity assessment of KRH-2 and KRH-4, seed lots F_1 hybrids were obtained from KSSOCA, Hebbal, Bengaluru. These seeds are deliberately mixed with the seeds of female parental line (CRMS 32A) in different proportion to create desired level of impurities.

Seeds of different genetic purity lots created artificially are indicated below:

KRH-2: L_1 and L_2: 100 per cent (assumed purity level) = 400 hybrid seeds

KRH-4: L_1: 100 per cent = 400 hybrid seeds

L_2: 98 per cent = 392 hybrid seeds+ 8 female parental seeds

L_3: 95 per cent = 380 hybrid seeds+ 20 female parental seeds

DNA isolation and PCR analysis

The purpose of marker analysis, total DNA was extracted from freshly germinated young seedlings of parental lines and hybrids (KRH-2 and KRH-4) following the protocol of Venderbeck *et al.* (1992). Quantification of DNA was accomplished by analyzing the DNA on 0.8 per cent Agarose gel using diluted uncut lamda DNA as standard and also by using nanodrop method. A total of 84 SSR primer pairs were used for PCR amplification. DNA samples (50 ng) were amplified in 15 µl of reaction volumes containing 10 x PCR buffer, 25 mM Magnesium chloride, Taq DNA polymerase (5 U / µl), 1 mM dNTPs and 0.5 µM primer. The basic profile was 5 min at 94^0C, 35 cycles of 30 sec at 94^0C, 30 sec at 58^0C, 1min at 72^0C and 5 min at 72^0C for final extension. The annealing temperature was adjusted based on the specific requirement of each primer combination. The PCR product was electrophoresed on 3 per cent Agarose gel. For genetic purity testing of KRH-2 and KRH-4 hybrids, DNA was isolated from 400 individual seedlings of three different seed lots (L_1, L_2 and L_3) and genotyping of these individual seedlings were performed by identified informative SSR markers.

For purity assessment of hybrid KRH-2, an SSR marker RM 164, which is polymorphic between IR-58025A and KMR-3 amplifying with an amplicon of 230bp and 200bp, respectively, was used.

For assesment of purity hybrid KRH-4 an SSR marker RM444, which is polymorphic between CRMS-32A and MSN-36, amplifying with an amplicon of 140bp and 170bp, respectively, was used.

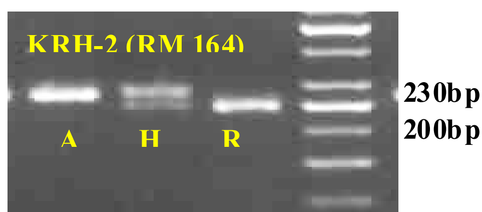
Results and Discussion

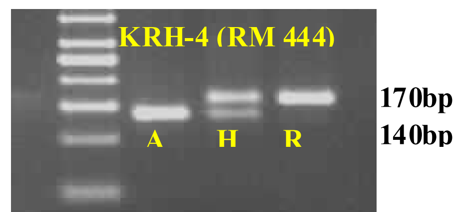
Identification of informative SSR markers for KRH-2 (IR-58025A and KMR-3) and KRH-4 (CRMS 32A and MSN 36) hybrids and assessment of genetic purity

The hybrids and their parental lines were characterized using 84 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 a hybrid when polymorphism was detected between the CMS and restorer lines. Among the 84 SSR primers, all of them generated PCR products of unusual base pairs. Out of 84 SSR primers studied RM 206, RM 276, RM 204, RM 202, RM 263, RM 216, RM 219, RM6844, RM1385, RM228, RM6696, RM21 RM209, RM7279, RM 9, RM 242 were identified earlier and RM 346, RM 400, RM 444 and RM 336 were newly identified and these markers were found to be polymorphic primers for KRH-4 (Plate 1) and its parental lines whereas RM 206 and RM 276 for hybrid rice KRH-2, RM 263 and RM 164 were newly identified markers.

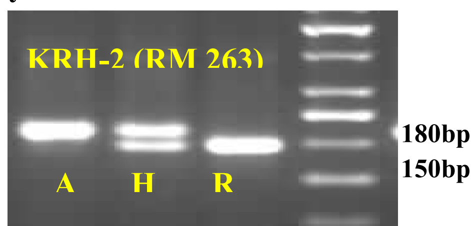
Accurate detection of impurities in seed it is essential to identify a set of informative markers which clearly distinguish the parental lines and amplify the unique allele combinations in the hybrids (Sundaram *et al.,* 2008). An informative markers for KRH-4 hybrid, SSR primers which are polymorphic for both KRH-2 and KRH-4 were compared to the polymorphic markers of other hybrids such as DRRH-1, DRRH-2, CORH-1, CORH-1, CORH-2 and CNRH-3 (Sundaram *et al.,* 2008; Tamilkumar *et al.,* 2009; Kumar *et al.,* 2015; Nethra *et al.,*2016 and Bora *et al.,* 2016). Most of the polymorphic markers of KRH-2 and KRH-4 were common to polymorphic markers of other hybrids (Table I). These common markers could not able to distinguish the hybrids from other when they are mixed either with KRH-2 and KRH-4.Since these were polymorphic SSR primers amplified similar allele for hybrids mentioned. Two SSR markers, RM-164 and RM-206 found very ‘specific’ and ‘unique’ to KRH-2. However, for KRH-4 hybrid, the unique informative markers such as RM 21, RM

Newly identified





Previously identified



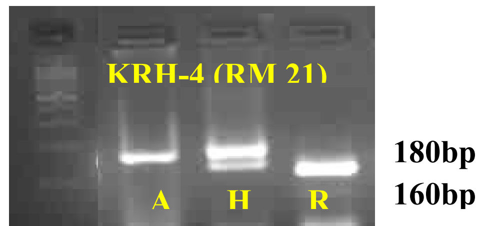


Plate 1: Comparison of newly identified and existing SSR markers of hybrid rice (KRH-2: RM 164 & RM 263; KRH-4: RM 444 & RM 21)

444 and RM 1385 could distinguish from other hybrids when there is an admixture of KRH-4 inadvertently.

Newly identified polymorphic SSR marker RM164 was used for purity assessment of KRH-2 hybrid. DNA was isolated from 20 days old seedlings of the 400 coded individual plants from each seed lots (L_1 and L_2) planted in green house condition in a 20 × 20 matrix and genotyping of these 400 individual seedlings was done using the polymorphic marker RM164. As expected two alleles (Hybrid) was present in all the lanes of 400 samples of L_1 lot and L_2 lot indicating 100 per cent purity of the seed lot (Plate 2). Thus an informative SSR marker RM164 effectively detected different percent of IR-58025A admixtures in KRH-2 (H) see lots ($L_1$and L_2) and thus RM164 can be efficiently used for hybrid purity assessment of KRH-2 seed lots.

Genetic purity testing of KRH-4 hybrid seed lot

Newly identified polymorphic SSR marker RM444 was used for purity assessment of KRH-4

TABLE I

Comparison of polymorphic markers for KRH-2 and KRH-4 with other public rice hybrids

Hybrids	Common polymorphic SSR markers of KRH - 4 with the following hybirds
KRH-2	RM 219, RM 206, RM 228, RM 202, RM 209, RM 276, RM 263
KRH-4	RM 206, RM 276, RM202, RM 204, RM 263, RM 216, RM 219, RM 6844, RM 1385, RM228, RM 6696, RM 21, RM 209, RM 400, RM 444, RM9, RM 242
DRRH-1	RM 219, RM 206, RM 336, RM 276, RM 216, RM 263, RM 6844
DRRH-2	RM 206, RM 276, RM 219, RM 204, RM 216, RM 228, RM 7279
CORH-1	RM 206, RM 216, RM 263
CORH-2	RM 336, RM 276, RM 206, RM 216 RM 258, RM 263, RM 6844
CORH-3	RM 263, RM 206, RM 216
	Unique markers identified for UASB hybrids
KRH-2	RM206, RM 164
KRH-4	RM 21, RM 1385, RM 444

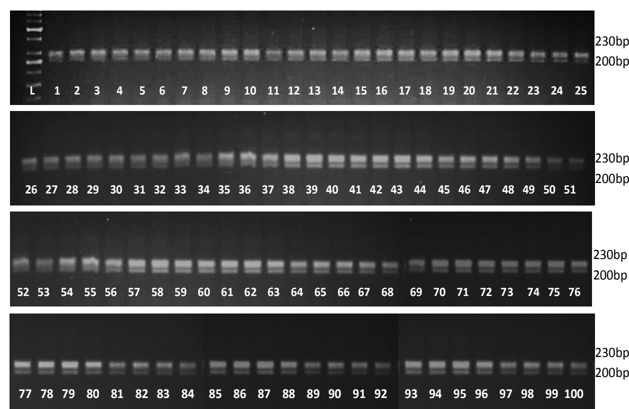


Plate 2 : SSR marker (RM 164) profile of seed lot (L_1) with 100 per cent genetic purity in rice hybrid KRH-2 (Lane No. 1 to 100 individual seed tested)

hybrid. DNA was isolated from 20 days old seedlings of the 400 coded individual plants from each seed lots (L_1 and L_2) planted in green house condition in a 20 × 20 matrix and genotyping of these 400 individual seedlings was done using the polymorphic marker RM444. As expected two alleles (Hybrid) was present in all the lanes of 400 samples of L_1 lot indicating 100 per cent purityof the lot (Plate 3). Whereas,in L_2 lot

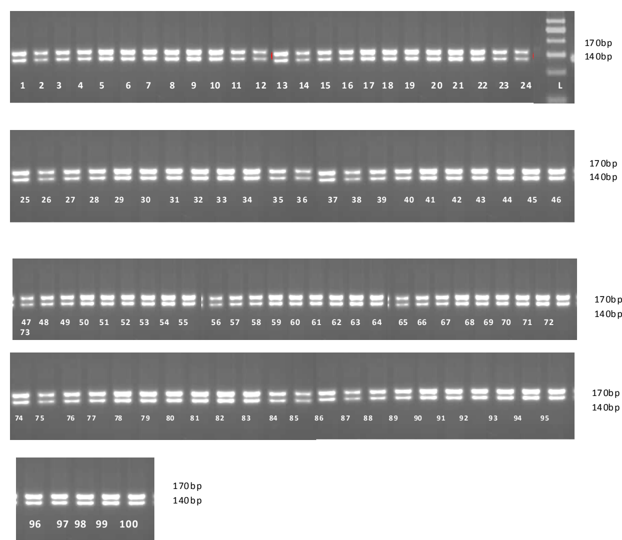


Plate 3 : SSR marker (RM 444) profile of seed lot (L_1) with 100 per cent genetic purity in rice hybrid KRH-4 (Lane No. 1 to 100 individual seed tested)

one allele was present in 20 samples hence, we can clearly identify the female line (CRMS 32A) contamination present in the hybrid KRH-4 seed lot and two alleles were present in 380 samples which indicates the 95 per cent purity of the seed lot (Plate 4). Thus an informative SSR marker RM444 effectively detected different percent of CRMS-32A admixtures in KRH-4 see lots (L_1 and L_2) and thus RM444 can be efficiently used for hybrid purity assessment of KRH-4 seed lots along with earlier identified markers for the genetic purity assessment.

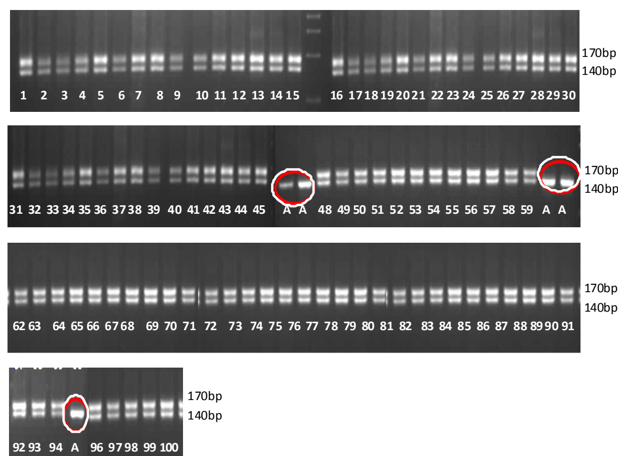


Plate 4 : SSR marker (RM 444) profile of seed lot (L_2) with 95 per cent genetic purity in rice hybrid KRH-4 (Lane No. 1 to 100 individual seed tested)

Genetic purity of a variety / hybrid plays a pivotal role in ensuring the agronomic performance and complete heterotic potential (Tamilkumar *et al*., 2009). Beside the stability of a variety bred for a particular trait can be maintained for several generations if the purity of a variety is assured. Purity of variety / hybrid is usually assured based on GOTs which rely on a set of morphological traits. However, these traits are likely to be, environmentally influenced and it requires more time compared with molecular markers which is rapid and highly accurate. Thus an informative SSR marker can be used to detect the level of contamination effectively in seed lots of hybrids KRH2 and KRH-4. Hence, RM 206, RM164 markers for KRH-2 and for KRH-4 RM 21, RM 444, RM1385 markers can be efficiently used for hybrid purity assessment of commercial seed lots. Therefore, considering the innate advantages, the marker based seed purity assay could be an alternative tool to grow out test which helps in detection of off types very rapidly and precisely without any ambiguity.

The DNA finger printing technology was found promising to differentiate the hybrids more accurately and efficiently from its parental lines and off types using informative SSR markers. Therefore, genetic

purity analysis through SSR markers could be an effective tool for rapid assessment of genetic purity of seed lots in hybrid rice and resolving problems arises due to natural genetic contamination in seed certification program. In addition, a molecular database of the rice cultivars would help breeders in the selection of parents in backcross breeding programmes. Although the cost of SSR marker tests is the major limitation, it is rapid and highly accurate compared with GOTs.

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