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Fingerprinting of Rice Hybrids and their Parental Lines using Microsatellite Markers and their Utilization in Genetic Purity Assessment of Hybrid Rice

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Abstract: Varietal identification has attained critical importance worldwide especially in the context of plant variety protection. The present investigation was undertaken with an objective to identify distinguishable microsatellite markers to establish fingerprinting of rice (*Oryza sativa* L.) hybrids, assessing variation within parental lines and testing the genetic purity of hybrid seed lot CORH3. About 11 most informative microsatellite markers were employed for fingerprinting five rice hybrids and their parental lines. A total of 32 alleles were obtained using 11 microsatellite primer pairs with an average of 2.90 alleles per primer pair. The number of alleles amplified for each primer pair ranged from 2 to 4. Nevertheless, five microsatellite markers together differentiated all the 5 hybrids and the parental lines. The microsatellite marker, RM234 amplified alleles specific to differentiate parental lines of CORH3 likewise RM 276 for KRH2, RM258 for PRH10, RM202 for AJAY and RM204 for RAJALAXMI used to differentiate parental lines of respective hybrids. The unique microsatellite marker, RM234 was used for testing the genetic purity of CORH3 seeds.

Key words: Fingerprinting, genetic purity, grow-out test, rice hybrids, microsatellite

INTRODUCTION

Rice is an important food crop with wide adaptation to a range of environments (Liu *et al.*, 2007). Hybrid rice cultivation offers an opportunity to increase rice yields and thereby ensures a steady supply of rice (Virmani and Kumar, 2004). It has been estimated that 1% impurity in the hybrid seed brings down the potential yield of hybrid by about 100 kg ha⁻¹ (Mao *et al.*, 1996). The ability to distinguish and clearly identify the varieties of cultivated species is fundamental for the operational aspects in the seed trade. The new varieties developed in agricultural and horticultural crops should be distinct from other varieties; Internationally DUS (distinctness, uniformity and stability) testing is co-ordinated by the International Union for the Protection of New Varieties of Plants (UPOV), which produces Guidelines detailing lists of characters to be used for examination of different species.

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Conventionally, the genetic purity of the rice hybrids is assessed by the Grow-Out Test (GOT) which becomes time consuming and expensive, requiring large areas of land and skilled personnel often making subjective decisions (Cooke, 1995). However, grow-out test requires one full season thus precluding the immediate cultivation of the hybrid seed produced. In addition, locking up of the capital invested on hybrid seed production and additional expenditure incurred on storage of hybrid seed ultimately increases the hybrid seed cost. This limitation and the environmental dependence of the entire procedure can be managed effectively by employing the molecular markers. The Biochemical and Molecular Techniques group of the International Union for the Protection of New Varieties of Plants (UPOV) is evaluating different DNA marker parameters prior to its routine use in establishing Distinctness, Uniformity and Stability (DUS) of plant varieties (Bredemeijer *et al.*, 2002).

Among the various markers available the co-dominant markers can differentiate between the homozygous and heterozygous genotypes. Therefore, it has been applied widely in the identification, registration of plant variety and in monitoring the seed purity and the authenticity with high accuracy, high reliability and low cost.

Among PCR based markers in rice, microsatellites are abundant and well distributed throughout the genome (Akagi *et al.*, 1996; McCouch *et al.*, 1997; Wu and Tanksley, 1993). They are valuable as genetic markers because they are co-dominant, detect high levels of allelic diversity and are assayed efficiently by the Polymerase Chain Reaction (PCR) (McCouch *et al.*, 1997). The current level of average genome-wide coverage provided by microsatellites in rice, one marker for every 6 centimorgans (Temnykh *et al.*, 2000) is sufficient to be useful for assessment of hybrid seed purity and for genotype identification. More than 2200 microsatellite markers have been mapped to specific locations in rice genome (McCouch *et al.*, 2002).

The use of SSR markers for genetic purity test been reported earlier in the commercial hybrid of PRH10 (Nandakumar *et al.*, 2004). The present study was formulated in order to identify a set of SSR markers for fingerprinting of five rice hybrids and their parental lines released recently and which are widely used in commercial scale in the seed chain. Further, the utility of these markers will be validated for genetic purity test using the commercial seed lot in order to determine the purity of seeds lot and compared with the GOT procedure which is being followed widely at present.

MATERIALS AND METHODS

Five public sector rice hybrids released for commercial cultivation in different parts of India and their parental lines were taken for the study (Table 1). Seeds of all genotypes were grown in field condition at Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu. A random sample of 450 seeds of CORH3 representing the commercial F₁ seed lot, produced at Paddy Breeding Station, Tamil Nadu Agricultural University, Coimbatore was used for

Table 1: Details of hybrids and their parental lines used in the study

S. No.	Hybrids	Parentage	
		CMS line	Restorer line
1	CORH3	TNAUCMS 2A	CB87R
2	KRH2	IR58025A	KMR3R
3	PRH10	PUSA6A	PRR78
4	CRHR7(AJAY)	CRMS31A	IR42266-29-3R
5	CRHR5(RAJALAXMI)	CRMS32A	IR42266-29-3R

testing their genetic purity both GOT and the DNA marker studies. The GOT was conducted at Wet lands of Central farm, Tamil Nadu Agricultural University, Coimbatore, during Rabi-2008.

SSR Analysis

Total DNA was extracted from fresh leaves by the Cetyl Tri-methyl Ammonium Bromide (CTAB) method (Murray and Thompson, 1980). The quality and concentration of extracted DNA were estimated by using a Nanodrop spectrophotometer. DNA was diluted in TE buffer for PCR analysis. About 11 most informative markers viz., RM234(7), RM276(6), RM164(5), RM70(7), RM247(12), RM204(6), RM202(11), RM216(10), RM1(1), RM258(10) and RM226(1) (where, the figures in the parenthesis refers to the chromosomal location of the respective markers) distributed in different chromosome of rice were selected for parental polymorphism survey. The primer sequence information was obtained from the published sequence data (www.gramene.org).

DNA amplification was carried out in a 15 μ L reaction volume containing 0.2 μ M of each primer, 200 μ M of deoxyribonucleotides, 50 mM KCl, 10 mM Tris HCl (pH 8.3), 1.5 mM MgCl₂, 0.1% gelatine, 40 ng of DNA and 0.5 unit of Taq DNA polymerase. The temperature profile used for PCR amplification comprised 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min and ending up with 5 min at 72°C for the final extension. The annealing temperature was adjusted based on the specific requirements of each primer combination. The PCR products were electrophoresed in 3% agarose gels at 100 V for 2 h. The gels were next stained in ethidium bromide for 30 min, de-stained for 15-30 min and then observed under a UV transilluminator.

RESULTS

Fingerprinting of Rice Hybrids and Their Parental Lines

The analysis using 11 microsatellite markers (RM234, RM276, RM164, RM70, RM247, RM204, RM202, RM216, RM1, RM258 and RM226) evenly distributed hyper polymorphic SSR markers allowed to identify several markers, which exhibited amplification of alleles specific or unique to a particular parental line which were used for fingerprinting the hybrids. A total of 32 alleles were obtained using 11 SSR primer pairs with an average of 2.90 alleles per primer pair. The number of alleles amplified for each primer pair ranged from 2 to 4. The markers RM204 and RM226 generated a maximum number of four alleles; another six SSR markers (RM202, RM234, RM276, RM164, RM70 and RM247) generated three alleles while rest of the markers generated two alleles.

Nevertheless, five microsatellite markers RM276, RM234, RM258, RM202 and RM204 together differentiated all the 5 hybrids and the parental lines at least with a single marker allele difference. The microsatellite marker, RM234 amplified alleles specific to differentiate parental lines of CORH3 likewise RM276 for KRH2, RM258 for PRH10, RM202 for AJAY and RM204 for RAJALAXMI used to differentiate parental lines of respective hybrids. The hybrid profile based on the five microsatellite markers which serve as distinct molecular tags are presented in Fig. 1. Apart from RM276, the rice hybrid KRH2 was also distinguished from other hybrids by using RM226 marker with the ~290 bp allele from KMR3R (Fig. 2). RM206 exhibited polymorphism for the parental lines of CORH3 and KRH2 with the same allelic pattern. RM202 was found to be heterozygous for PRH10, AJAY and RAJALAXMI. It distinguished the Cuttack hybrids (AJAY and RAJALAXMI) from others (Fig. 3). The SSR marker alleles identified as molecular tags for distinguishing the rice hybrids and their parental lines are presented in Table 2.

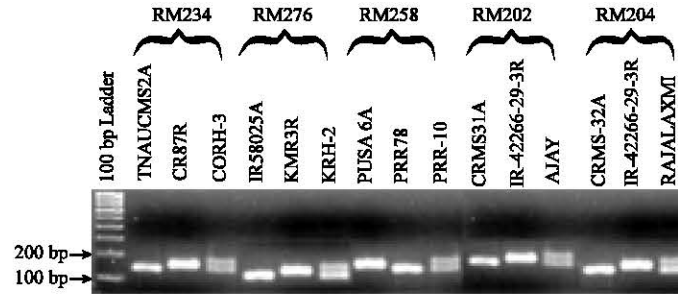


Fig. 1: Hybridity microsatellite markers polymorphism between parental lines and rice hybrids

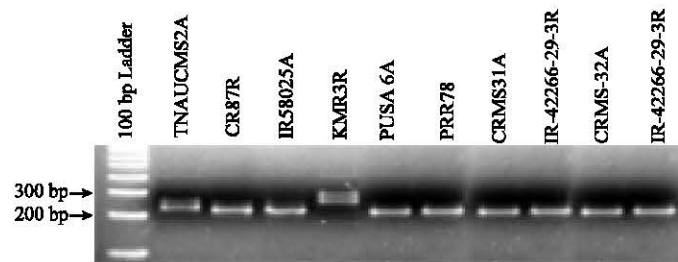


Fig. 2: Amplification pattern of the parental lines obtained using the SSR marker RM226

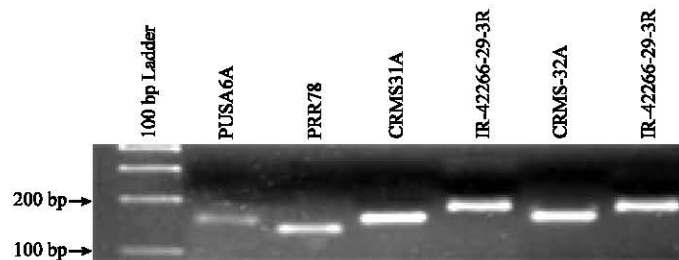


Fig. 3: Amplification pattern of the parental lines obtained using the SSR marker RM202

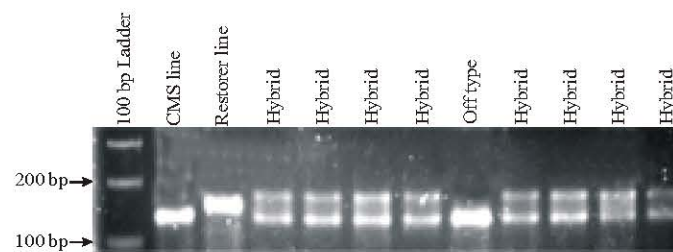


Fig. 4: Testing genetic purity of hybrid seeds of CORH3 using the SSR marker RM234, Lane 2: TNAUCMS2A (CMS line), Lane 3: CB87R (restorer line). DNA was isolated from single seedlings of the CORH3 hybrid, PCR analysis was performed and genotype assessed (Lanes 4-12) as described in Materials and Methods. Off type in Lanes 8

Table 2: SSR marker alleles identified as molecular tags for rice hybrids using single marker

SSR marker	Hybrid	Size of allele (bp)	
		CMS line	Restorer line
RM234	CORH3	145	170
	RAJALAXMI	135	150
RM 276	KRH2	120	140
RM226	KRH2	240	290
RM 258	PRH10	170	160
RM 202	AJAY	175	182
RM 204	RAJALAXMI	115	130

Testing Genetic Purity of Hybrid Seeds

Assessment and maintenance of genetic purity of hybrids play crucial role in successful popularization and adoption of hybrid rice technology. But there are several ways for chance of contamination in the hybrid seed production plot due to pollen shedders, out crossing and physical mixtures during the post harvest operation. For testing genetic purity of CORH3 seeds SSR marker RM234 which is specific polymorphic between TNAUCMS2A and CB87R with marker amplified ~145 and ~170 bp fragments in TNAUCMS2A and CB87R was used. Genomic DNA was isolated from 50 seedlings of the CORH3 hybrid PCR analysis was performed by means of the RM234 out of 50 random samples microsatellite marker identified presence of single pollen shedder (B line) seed, which had a CMS line specific fragment (Fig. 4). This amounts to 2% off types in the hybrid seed produced. The results were confirmed using 400 seeds from the same seed lot through Grow out Test (GOT).

DISCUSSION

Varietal identification and purity testing assumes greater importance in new IPR issues. Distribution of genetically pure good quality seed to farmers will facilitate complete heterotic expression of hybrids in rice. The finger printing of rice hybrids and their respective parental lines and testing genetic purity of rice hybrids using microsatellite markers are discussed in the present study. Possible application of DNA profiling techniques for plant variety registration and plant breeders rights (DUS testing) is being studied worldwide (Lee *et al.*, 1996). The various commercial hybrids and parental lines of rice hybrids were hence subjected to molecular marker analysis for genetic purity test. The stability of SSR markers over different environments, no stage specificity and the advent of rapid and workable techniques make molecular techniques convenient for testing distinctness of varieties and also for future protection (Law *et al.*, 1998). Further SSR markers have good characters like reproducibility, multiallelic nature, co-dominant inheritance, relative abundance and good genome coverage (Powell *et al.*, 1996). Microsatellite DNA markers based on Simple Sequence Repeats (SSRs) as amplified by PCR have been used successfully as tools for varietal identification (Yang *et al.*, 1994; Rongwen *et al.*, 1995). In the present investigation, 11 SSR primer pairs amplified a total of 32 alleles ranging from 2 to 4 alleles per primer. The average number of alleles per primer was 2.90. Because of the polyallelic nature the SSR markers have the advantage of discriminating the individuals more precisely. Similar results were reported in different rice hybrids by earlier workers (Nandakumar *et al.*, 2004).

In case of hybrids, F₁ seeds are commercially grown by farmers, which make it necessary to use the fresh seed every year. Although, the hybrids are costly, the farmers grow hybrids because of higher yield and the overall high economic return. The commercial success of

hybrid technology depends to a large extent on the quality of the hybrid seed supplied, especially the genetic purity. Therefore, the molecular fingerprinting of the CMS lines, restorer lines and the hybrids assumes utmost importance for protecting Plant Breeders Rights on them and ensuring genetic purity.

The only legally recognized traditional method in our country for genetic purity assessment continues to be seed certification based on field plot grow out tests, which include only the morphological characteristics of a variety and these traits are often inadequate and suffers due to environmental influences. In addition, morphological plant evaluation is deficient in assessing the genetic purity of seed sample due to environmental effects of morphological traits (Cooke, 1999). Further, the morphological traits are multigenic or quantitative and their expression is altered by environmental factors, requires replications of observations, trained personnel, suitable land and is time consuming. This limitation and the environmental dependence of the entire procedure can be managed effectively by employing the molecular markers. Many studies have shown that SSR markers are useful in identification of rice hybrids and their respective parents, assessment of plant to plant variation within parental lines and testing the genetic purity of rice hybrids (Yashitola *et al.*, 2002; Nandakumar *et al.*, 2004; Yun *et al.*, 2005; Sundaram *et al.*, 2007). Through this study the utility of the microsatellite marker, RM234 was evident which amplified alleles specific to differentiate parental lines of CORH3 for testing the genetic purity.

These molecular markers effectively differentiate the hybrids from off types mainly pollen shedders (presence of B line in the A line) in the hybrid seed lot. Pollen shedder is the major factor affecting genetic purity of hybrid rice seed production. Using molecular marker for testing genetic purity substantially reduce the time, space, labour and ultimately the cost involved in testing the genetic purity of hybrid seeds (Nandakumar *et al.*, 2004; Sundaram *et al.*, 2007). Therefore, it is concluded that genetic purity analysis through SSR marker will be a useful tool for resolving the problem arises in seed certification programme as well as the rapid determination of genetic purity of the rice hybrids.

CONCLUSION

The success of hybrid rice technology beside other factors depends on the production and timely supply of genetically homogenous seeds to farmers. The present study establishes the microsatellite marker, RM234 amplified alleles specific to differentiate parental lines of CORH3 likewise RM 276 for KRH2, RM258 for PRH10, RM202 for AJAY and RM204 for RAJALAXMI used to differentiate parental lines of respective hybrids. The present study also reveals testing the genetic purity of CORH3 seeds with unique microsatellite marker, RM234. The high discriminating power of SSR markers and inexpensive setup should allow this to be affordable for hybrid purity assessment.

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