Electronic Journal of Plant Breeding, 4(1): 1021-1026 (Mar 2013)
ISSN 0975-928X

Research Article

Hybrid purity testing in rice (*Oryza sativa L.)* using microsatellite markers

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(Received: 08 Dec 2012; Accepted: 16 Mar 2013)

Abstract
Microsatellite markers are useful in fingerprinting, for assessing variation within parental lines and testing the genetic purity of hybrid seed in rice. In this paper we report the identification of pure rice hybrids using microsatellite polymorphisms. Thirty simple sequence repeat microsatellite (SSR) markers were employed for fingerprinting 18 rice hybrids and their parental lines. Twelve SSR markers were found polymorphic across the parents and produced unique fingerprint for the parents. Among the markers RM-17 and RM-84 precisely distinguished between pure hybrids and mixture/off type. Cluster analysis based on Simple matching (SM) similarity coefficient using UPGMA grouped the hybrids into three clusters. The genetic similarity between the hybrids ranged from 0.43 to 0.81 with an average similarity index of 0.63.

Keywords:
Rice, marker, hybrid purity, GOT

Introduction

Rice is the principal food crop, feeding more than half of the world's population (Virmani, 1999). Ever since the report of Jones (1926), exploitation of heterosis has been contemplated as a potential strategy for yield enhancement in most of the crops, including rice, which became a reality after the commercial success in China. At present, hybrid rice is commercially cultivated in China, India, Vietnam and Philippines. India, a predominantly rice growing country has released many hybrids including a superfine grain aromatic rice hybrid for commercial cultivation. At present, the hybrid seed production in rice is primarily based on three-line system, which involves a cytoplasmic male sterile (CMS) line or A line, a corresponding iso-nuclear maintainer (B) line and a genetically diverse restorer (R) line. The A line is maintained by crossing it with B line and hybrid seed is produced by crossing A line with R line. Assessment and maintenance of genetic purity of the parental lines and hybrids is crucial in delivering the pure hybrid seed for the successful adoption of this technology. Molecular markers have the potential in achieving this goal (Jena and Pandey, 1999; Yashitola *et al*., 2002). Unambiguous identification of elite crop varieties and hybrids is essential for their protection and prevention of unauthorized commercial use. A set of qualitative and quantitative characters, known as descriptors, are currently in use for variety identification and description. Some of these characters, particularly those showing quantitative inheritance, interact with the environment in which the variety is grown and thus make the process of variety identification subjective. Molecular markers, in contrast, being based on DNA sequence variation, provide an unbiased means of identifying crop varieties (Nandakumar *et al*., 2004, Tamilkumar et al., 2009, Xin et al., 2005). Among the various DNA based markers currently available, genetically mapped Simple Sequence Repeats (SSR) are the markers of choice in rice because of their abundance, co-dominant nature and uniform distribution throughout the genome (McCouch *et al.*, 1997). The primary objective of the present study was to identify the rice hybrids and differentiation of their parental lines by employing SSR markers.

Material and methods

Plant materials: For the purpose of identifying the purity of hybrid seeds, known parents *viz*., 3 CMS lines namely, IR 58025A, IR 79156A (IRRI, Philippines) and CRMS 31A (CRRI, Cuttack) 6 restorer lines OR2310-12 (Orissa), R-1213-3 (IGKV, Raipur), R1060-1674-1-1 (IGKV, Raipur), Jhitpiti (Germplasm, IGKV, Raipur), PR-111 (PAU, Punjab), P-1460 (IARI, New Delhi) were obtained from the Department of Genetics and Plant Breeding, Indira Gandhi Krishi Vishwavidyalaya, Raipur. For the purpose of marker analysis, seeds were germinated using sterile media under aseptic condition and were crossed during *Rabi* 2010-11 and F_1 seeds were harvested.

Molecular analysis: Plant DNA was isolated using mini prep method. Quantification of DNA was accomplished by Nanodrop Spectrophotometer at OD_{260nm}. DNA was diluted in $Tris_{10}$:$EDTA_1$ buffer to a concentration of approximately 20 ng/µL for PCR analysis. 30 SSR microsatellite primers with known sequences were analyzed, of which only 12 markers *viz.*, RM-17 (12), RM- 84 (1), RM-201 (9), RM-212 (1), RM-220 (1), RM-277 (12), RM-278 (9), RM-411 (3), RM-433 (8), RM-506 (8), RM-524 () and RM-566 (9) (where, the figures in the parenthesis refers to the chromosomal location of the respective markers) showed parental polymorphism. DNA amplification was carried out in 20 µL reaction mixture containing 10X PCR buffer 2.0 µl, 15 mM $MgCl_2$ 0.5 µl, dNTPs (Mix) 2.5 mM 0.5 µl, Primer (forward) 5 Pmol 0.5 µl,

Primer (reverse) 5 Pmol 0.5 µl, *Taq* polymerase 1 unit / µl 0.5, DNA template 20ηg/µl 4.0 µl and nano pure water 11.5 µl. The amplification reaction was carried out in Applied Biosystem Veriti 96 well Thermal cycler. The first cycle consists of denaturation of template DNA at 95^{o}C for 4 minutes, primer annealing (55^{o}C) for 1minute and primer extension (72^{o}C) for 1minute. In next 32 cycles, the period of denaturation was reduced to 1minute while the primer annealing and primer extension time remained as in the first cycle. The last cycle consists only of primer extension (72^{o}C) for 7 minutes. PCR products were mixed with 3µl of 6X loading and were separated on 5% PAGE gel using 1 X TBE buffer. The size of the amplified fragments was determined by using size standards (50 bp DNA ladder, GeNei, Bangalore). Electrophoresis was done for sufficient time (usually 40-60 minutes) minutes at 180 volts. Gels were further stained by Ethidium Bromide staining method and the DNA banding pattern of the samples was visualized through BIORAD UV Gel doc XR+ system.

Cluster analysis: The amplified products were scored as present (1) or absent (0) for each primer. Cluster analysis was performed using Simple Matching (SM) coefficient of similarity matrices calculated from SSR markers to generate a dendrogram of nine parental lines based on similarity coefficient using Unweighted Pair Group Method based on Arithmetic mean (UPGMA) using the NTSYS - pc software (Rohlf, 1998).

Results and discussion

The 18 rice hybrids and their parental lines were analyzed using microsatellite markers. The parental polymorphism survey indicated that out of 30 markers, 12 SSR markers (Table 2) showed parental polymorphism. The profile of SSR primer RM 17 is depicted in figure 1 which clearly shows parental polymorphism between the nine parents i.e., three CMS lines and six restorer lines. The band size varies from 150 to 200 bp. Similar profile was also observed with the marker RM 84 which also showed parental polymorphism between the A and R lines (Fig. 2).

Determining the purity of hybrid seed is an essential requirement for its commercial use since there is always a chance of contamination in the hybrid seed production plot because of pollen shedders, out crossing and physical mixtures during the subsequent handling of the harvested material. To determine the purity of hybrids banding pattern of hybrid plants and their parental lines were studied and found that pure hybrid gives two bands corresponds to their parental lines bands. Off types or mixtures shows differential banding pattern than the parental bands. To test the genetic purity of hybrids, RM 84 was the only marker which gave significant results between the parents and hybrids (Fig 2). IR79156A (A line) when crossed with Jhitpiti (R line) showed band size of 110 and 130 bp, respectively. The gel picture clearly depicts the presence of both parental bands in the F_1 hybrid (IR79156A x Jhitpiti). In another case, IR58025A (A line) when crossed with PR-111 (R line) also showed polymorphism with band size of 110 and 130 bp, respectively. The same case was again observed in CRMS31A (A line) when crossed with Jhitpiti**.** Overall, in all the crosses, F1 hybrids namely, IR79156A/JHITPITI, IR58025A/ PR-111 and CRMS31A/JHITPITI possessed the bands from both the parents i.e., A line and R line, indicating true F1 hybrids (Fig. 2).

Testing genetic purity of hybrid seeds: For testing the genetic purity of hybrid seeds, DNA from 20 plants of cross IR 79156A/ Jhitpiti was isolated and RM 84, SSR marker was used to check the purity along with the parents. The results clearly indicated that 19 out of 20 plants had both the parental bands except plant number 3 which, did not have any result because of non-amplification of PCR product. The band size in the parents and the population ranged between 60 to 70 bp (Fig. 3). SSR markers have been used successfully for variety identification (Yang et al, 1994, Rahman et al., 2009, and testing the genetic purity of rice hybrids (Nandakumar et al., 2004, Sundaram et al., 2007, Tamilkumar et al., 2009).

Fingerprinting of parents: The parental lines were grouped into three major clusters (Figure 4). The similarity coefficients ranged from 0.43 to 0.81 with an average similarity index of 0.63. In pair-wise comparison, the maximum similarity was obtained between CMS line of CRMS31A and OR2310-12 with a similarity index of 0.81, whereas parental lines of R1060-1674-1-1showed least similarity with similarity index of 0.43 with rest of the parents. The average similarity index among the genotypes was low which indicated greater genetic diversity among the parental lines.

Cluster I consists of parental lines OR2310-12, CRMS31A, R-1213-3, JHITPITI, and IR58025A, where OR2310-12 is 81 per cent similar to CRMS31A, R-1213-3 is 75 per cent similar to JHITPITI and IR58025A is 66 per cent similar with R-1213-3 and JHITPITI. Cluster II consists of PR-111, IR79156A and P-1460, where PR-111 is 69 per cent similar to IR79156A and P-1460 is 56 per cent similar with PR-111and IR79156A. Cluster I and Cluster II are 51 per cent similar and Cluster III consists of a single parent R1060-1674-1-1 which is 43 per cent similar to rest of the parents.

Conclusion

Hybrids are generally known for their heterotic potential and are being commercially cultivated to

gain higher yield. To exploit the full potential of the hybrids, it is very much essential to supply genetically pure hybrid seeds. Assessing genetic purity of hybrids through grow out test is tedious and time consuming. Alternatively, microsatellite markers are considered more reliable due to their ability to produce high fidelity profile as a result of their co-dominant nature and chromosome specificity. In the present study 12 polymorphic markers clearly distinguish all the parental lines. Out of these markers RM-17and RM-84 distinguish pure hybrids and mixture/off types very precisely. However, all the 12 markers are still relevant and can be used to determine the purity of rice hybrids.

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Table 1: Details of parents and their hybrids used in study

S. No.	CMS Line/ A Line	Restorer Line/ R Line	Hybrid/ F_1 (A line X R Line)
1	IR 58025A	OR2310-12	IR 58025A X OR2310-12
2	IR 58025A	R-1213-3	IR 58025A X R-1213-3
3	IR 58025A	R1060-1674-1-1	IR 58025A X R1060-1674-1-1
4	IR 58025A	Jhitpiti	IR 58025A X Jhitpiti
5	IR 58025A	PR-111	IR 58025A X PR-111
6	IR 58025A	P-1460	IR 58025A X P-1460
7	IR 79156A	OR2310-12	IR 79156A X OR2310-12
8	IR 79156A	R-1213-3	IR 79156A X R-1213-3
9	IR 79156A	R1060-1674-1-1	IR 79156A X R1060-1674-1-1
10	IR 79156A	Jhitpiti	IR 79156A X Jhitpiti
11	IR 79156A	PR-111	IR 79156A X PR-111
12	IR 79156A	P-1460	IR 79156A X P-1460
13	CRMS 31A	OR2310-12	CRMS 31A X OR2310-12
14	CRMS 31A	R-1213-3	CRMS 31A X R-1213-3
15	CRMS 31A	R1060-1674-1-1	CRMS 31A X R1060-1674-1-1
16	CRMS 31A	Jhitpiti	CRMS 31A X Jhitpiti
17	CRMS 31A	PR-111	CRMS 31A X PR-111
18	CRMS 31A	P-1460	CRMS 31A X P-1460

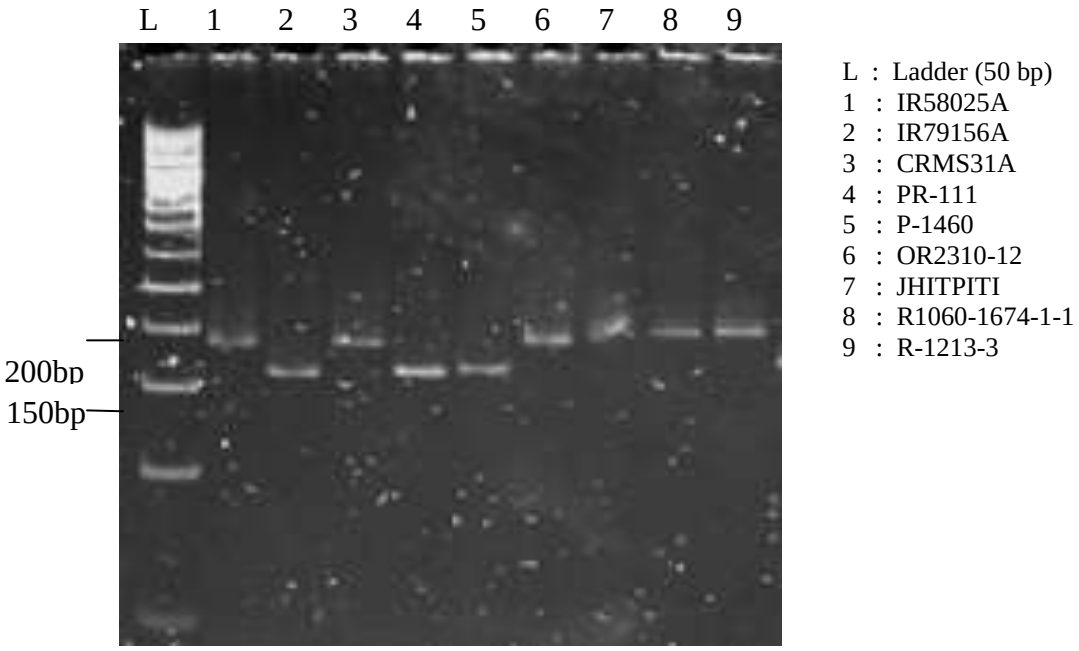


Figure 1. SSR marker RM 17 showing parental polymorphism between A and R lines

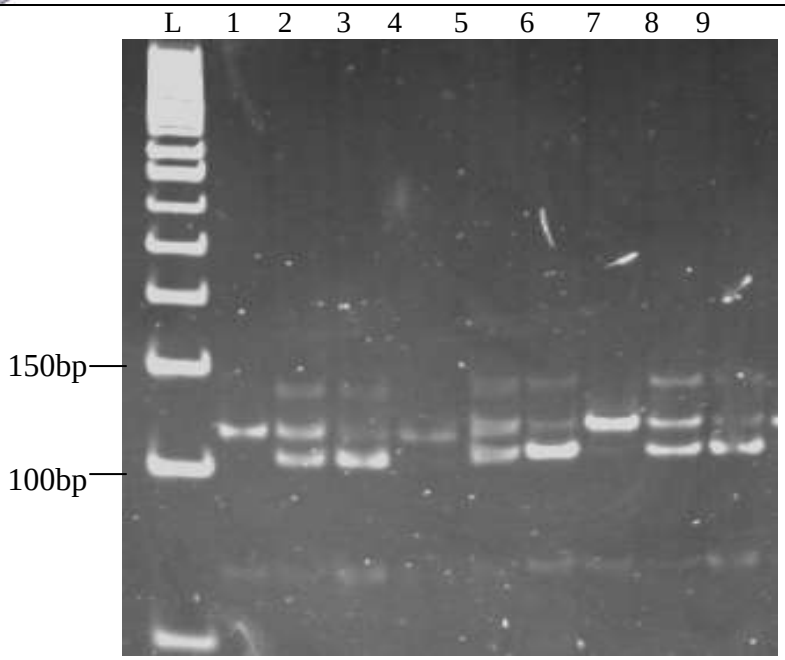
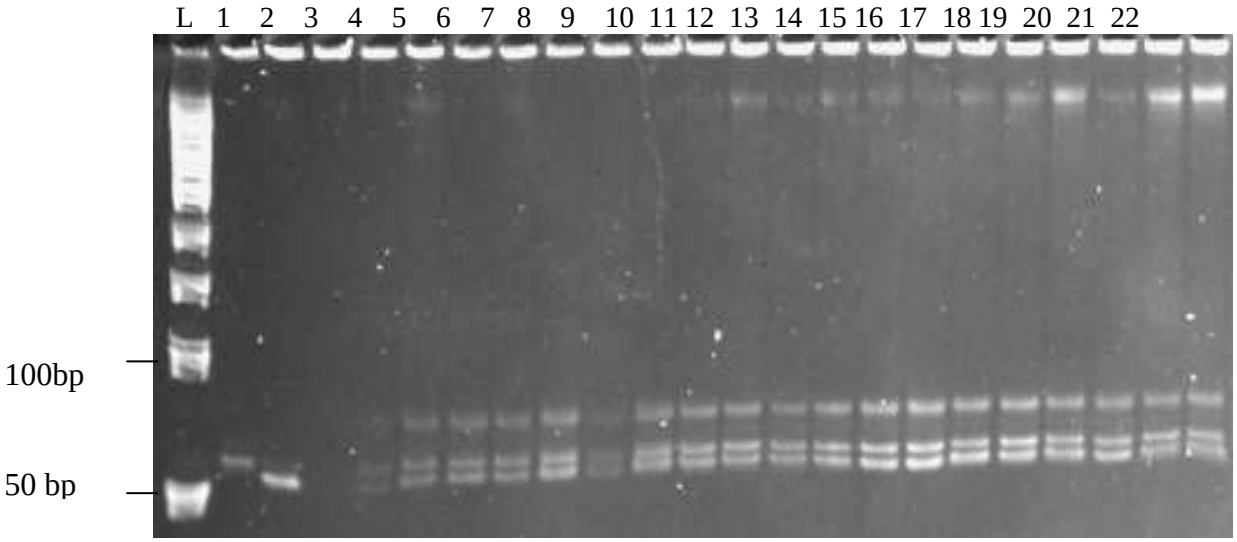


Figure 2. SSR marker RM 84 showing parental polymorphism in A line and R lines and hybrids



L: Ladder (50 bp), 1: IR79156A, 2: JHITPITI and 3 to 22 were hybrids (IR79156A /JHITPITI)

Figure 3. SSR marker RM 84 with 20 hybrid plants of IR79156A/JHITPITI

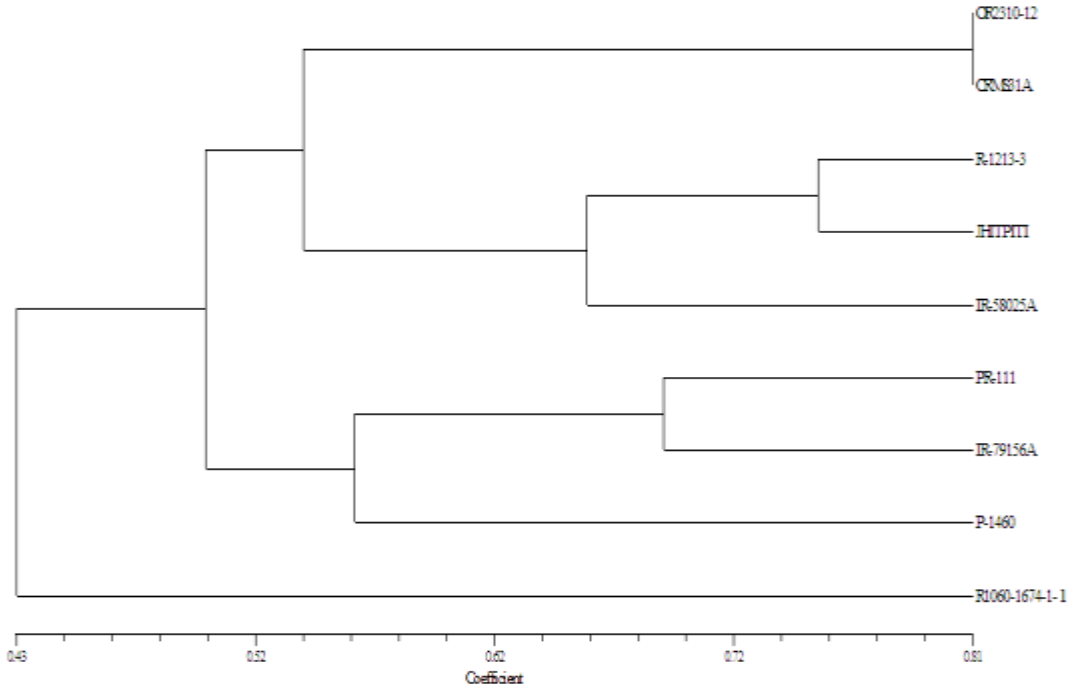


Figure 4. The dendrogram showing genetic relationship among nine parental lines