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Morphological characterization and assessment of genetic purity of rice hybrids using SSR markers

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ABSTRACT

Morphological and molecular markers were used for fingerprinting of hybrids, assessing variation within parental lines and testing of genetic purity of hybrid seed lot in rice. Conventionally, genetic purity of hybrids is assessed by grow out test (GOT). The distinguishing characters were used for the identification of hybrids from the lot, but it takes one full season. In order to reduce this time, molecular markers can be used as tools. In the present investigation, sixteen SSR markers were employed for finger printing of 3 rice hybrids and their parental lines. Eight primers amplified a maximum of two alleles whereas, all other were detected as monomorphic. Genetic purity of hybrid JRH 5, the SSR markers RM 234, RM 228, RM 84, RM 279 and RM 237 were identified. However, to test the genetic purity for JRH 8 and JRH 19, markers RM 234, RM 228, RM 279 and RM 510 were identified for testing the purity of these hybrids. Thus from this study it might be concluded that the molecular markers greatly augmented the entire process of testing genetic purity of hybrid seeds, by saving one full crop season and reducing the cost of hybrid seed.

Key words: *hybrid, genetic purity, SSR markers, parental lines*

Rice is one of the most important stable food crops of India (Singh *et al.,* 2014). Nearly 3 billion people almost daily consume it. The productivity of rice has now stagnated. Ever since the report of Jones (1926), exploitation of heterosis has been contemplated as a potential strategy for yield enhancement in rice, which became a reality after the commercial success of rice hybrid in China (Nandakumar *et al.,* 2004). Hybrid rice has clearly shown a standard heterosis of 15-20% in commercial cultivation mainly in the *indica* genotypes (Hossain *et al.,* 2010). Rice being self pollinated crop, use of male sterility is a pre-requisite for commercial exploitation of heterosis in rice. Cytoplasmic genetic male sterility/ three-line has been widely used for developing rice hybrids. This system involves a CMS or 'A' line, a corresponding iso-nuclear maintainer or 'B' line and genetically diverse restorer or 'R' line.

The hybrid seed is produced by crossing of male sterile line with restorer line. Assessment and maintenance of genetic purity of the parental lines and hybrids is necessary for successful adoption of hybrid rice technology. For this purpose, morphological characterization and molecular markers are used. A set of qualitative and quantitative characters as descriptor are currently in use for variety identification and description. The distinguish characters are used for the identification of purity of hybrids. But 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 identification subjective (Nandakumar *et al.,* 2004). Molecular markers have proven a potential tool in achieving this goal (Jena & Pandey, 1999; Yashitoal *et al.,* 2002). DNA markers are most widely used and predominant due to their abundance and more reproducibility and remain unaffected across different stages, seasons, locations and agronomic practices (McCouch *et al.,* 1997). Microsatellite or SSR markers are simple tandomly repeated, di to tetra nucleotide sequence motifs flanked by unique sequence. They are

valuable as genetic markers because they detect high level of allelic diversity, co dominant nature as well as easily and economically assayed by PCR (Jain *et al.*, 2004). The main objective of the present investigation was to identify rice hybrids and differentiation of their parental line using morphological and molecular (SSR) markers.

MATERIAL AND METHODS

Plant materials

For the purpose of morphological and molecular characterization, three rice hybrids released for commercial cultivation in different states of the country and their parental lines were used. The F_1 seeds and their parental lines were grown in randomized complete block design at Seed Breeding Farm, Department of Plant Breeding and Genetics, JNKVV, Jabalpur during wet season 2013 and 2014. Fourteen days old seedlings were transplanted in 5x2 m^2 plot with three replications. Distance between plant and between rows was 20 cm and 20 cm, respectively. Standard agronomic practices were followed throughout the crop growth period to obtain good harvest. Observation were recorded on five randomly selected plants from each replication, parents and hybrids for early plant vigour, basal leaf sheath colour, leaf blade colour, leaf hairs, apiculus colour, panicle type, seed coat colour, hull colour, threshability, aroma, plant height and days to 50 % flowering. For the purpose of molecular analysis, seeds were germinated using sterile media under aseptic condition (Table 1).

Isolation of genomic DNA

Genomic DNA from fresh leaves was isolated from parents and hybrids at seedling stage following the CTAB (cetyl trimethyl ammonium bromide) procedure as described by Saghai Maroof et al. (1984) with some modifications. Random primers were selected for hybrid purity testing (Table 1). Information regarding chromosomal location and repeat types can be found in web database (www.gramene.org). Quantification of DNA was accomplished by analyzing the DNA on 0.8% agarose gel stained with ethidium bromide using diluted uncut lambda DNA as standard.

PCR amplification

Polymerase chain reaction (PCR) was performed in a 10µl volume reaction mixture containing 2X PCR assay buffer, 1.5mM $MgCl_2$, 100µM of each dNTPs, 12ng each of forward and reverse primers, 0.2 units of Taq DNA polymerase and 25 ng of genomic DNA template. Amplification reaction initiated with a 5-minute pre-denaturation steps at 94^0 C followed by 35 cycles of DNA denaturation at 94^0 C for 30 seconds, primer

Table 1. List of primers used in fingerprinting of hybrids with their information

Primers	Reverse sequence	Forward sequence	Amplification temperature
RM 510	5 -AACCGGATTAGTTTCTCGCC-3	5 -AACCGGATTAGTTTCTCGCC-3	55
RM 237	5 -TGGGAAGAGAGCACTACAGC-3	5 -CAAATCCCGACTGCTGTCC-3	55
RM 455	5 -AGAAGGAAAAGGGCTCGATC-3	5 -AACAACCCACCACCTGTCTC-3	55
RM 137	5 -CGGGTCGTCCCCGAGGATCTTG-3	5 -GACATCGCCACCAGCCCACCAA-3	55
RM 428	5 -CGCTGCATCCACTACTGTTG-3	5 -AACAGATGGCATCGTCTTCC-3	55
RM 475	5 -ACGGTGGGATTAGACTGTGC-3	5 - CCTCCGATTTTCCTCCAAC-3	55
RM 202	5 - CCAGCAAGCATGTCAATGTA-3	5 - CAGATTGGAGATGAAGTCCTCC-3	55
RM 171	5 -AACGCGAGGACACGTACTTAC-3	5 -ACGAGATACGTACGCCTTTG-3	52
RM 234	5'-ACAGTATCCAAGGCCCTGG-3'	5'-CACGTGAGACAAAGACGGAG-3'	52
RM 84	5 - TTGCAAATGCAGCTAGAGTAC-3	5 - TAAGGGTCCATCCACAAGATG-3	52
RM 228	5 - GCTTGCGGCTCTGCTTAC-3	5 - CTGGCCATTAGTCCTTGG-3	52
RM 236	5'-GGCATCCCTCTTTGATTCCTC-3'	5'-GCGCTGGTGGAAAATGAG-3'	55
RM 85	5 - GCACAAGGTGAGCAGTCC-3	5 - CCAAAGATGAAACCTGGATTG-3	55
RM 241	5 - TGCAAGCAGCAGATTTAGTG-3	5 - GAGCCAAATAAGATCGCTGA-3	52
RM 488	5 - TAGCAACAACCAGCGTATGC-3	5 - CAGCTAGGGTTTTGAGGCTG-3	55
RM 42	5'-TTTGGTCTACGTGGCGTACA-3'	5'-ATCCTACCGCTGACCATGAG-3'	55
RM 237	5 -TGGGAAGAGAGCACTACAGC-3	5 -CAAATCCCGACTGCTGTCC-3	55

annealing at 50-55^0 C for 30 seconds and DNA extension at 72^0 C for 7 minutes was performed after 35 cycles. Amplified PCR products were separated on 2.5% of agarose gel at a voltage of 90V for a period of 45 minutes to 1 hour in 1X TBE buffer stained with ethidium bromide. The gel was visualized in UV trans-illuminator and photograph taken using Alpho Digidoc gel documentation instrument. Clearly resolved, unambiguous band were scored visually for their presence and absence with each primer by using size standard (100bp DNA ladder).

RESULT AND DISCUSSION

Morphological Characterization

The commercial success of hybrid rice technology depends to a large extent on the quality of hybrid seed supplied, especially the genetic purity. Conventionally the genetic purity of hybrids is assessed by the grow out test (GOT). The distinguish characters of all the three hybrids were presented in the Table 2-3.

However, GOT trial requires one full season precluding the immediate cultivation of the hybrid seed produced. In addition, the locking up the capital invested on hybrid seed production and additional expenditure incurred on storage of the produced hybrid seed ultimately increases the cost of hybrid seed production. This limitation and the environmental dependence of the entire procedure can be managed effectively by employing the molecular markers (Nanadakumar *et al.*, 2004) (Table 4-5).

Fingerprinting of rice hybrids

The three rice hybrids and their parental lines were analyzed for the microsatellite polymorphism. The parental polymorphism survey identified by the 16

Table 2. Distinguished morphological characters of hybrid JRH 5 and their parental lines

Characters	IR 68897 A (Female)	NPT 65 (Male)	JRH 5(Hybrid)
Plant height (cm)	95	110	95
Days to 50% flowering	88	100	70
Early plant vigour	Very good	Very good	Very good
Basal leaf colour	Green	Green	Dark green
Leaf blade colour	Green	Green	Dark green
Leaf hairs	Absent	Absent	Absent
Apiculus colour	White	White	White
Panicle type	Open	Open	Open
Seed coat colour	White	White	White
Hull colour	Straw	Straw	Straw
Aroma	Present	Absent	Absent
Threshability	Easy	Easy	Easy
Grain type	Long Slender	Medium Bold	Long slender

Table 3. Distinguished morphological characters of hybrid JRH 8 and their parental lines

Characters	IR 68897 A (Female)	NPT 29(Male)	JRH 8(Hybrid)
Plant height (cm)	82	90	85
Days to 50% flowering	85	95	75
Early plant vigour	Very good	Very good	Very good
Basal leaf colour	Green	Green	Dark green
Leaf blade colour	Green	Green	Dark green
Leaf hairs	Absent	Absent	Absent
Apiculus colour	White	White	White
Panicle type	Open	Open	Open
Seed coat colour	White	White	White
Hull colour	Straw	Straw	Straw
Aroma	Present	Absent	Absent
Thresh ability	Easy	Easy	Easy
Grain type	Long Slender	Medium Bold	Medium slender

Table 4. Distinguished morphological characters of hybrid JRH 19 and their parental lines

Characters	IR 58056A (Female)	NPT 13-01 (Male)	Hybrid
Plant height (cm)	83.28	115.72	84
Days to 50% flowering	94	115	75
Early plant vigour	Very good	Very good	Very good
Basal leaf colour	Green	Green	Dark green
Leaf blade colour	Green	Green	Dark green
Leaf hairs	Present	Present	Absent
Apiculus colour	Straw	Straw	White
Panicle type	Bunchy	Bunchy	Open
Seed coat colour	White	White	White
Hull colour	Yellow	Yellow	Straw
Aroma	Absent	Absent	Absent
Thresh ability	Easy	Difficult	Easy
Grain type	Long slender	Medium bold	Long slender

Table 5. List of the markers and their amplified fragments in all the three hybrids

Hybrid	Markers	Amplified fragments		
		CMS line	Restorer	Hybrid
JRH 5	RM 234	130 bp	150bp	130bp, 150bp
	RM 237	130 bp	120 bp	120 bp, 130 bp
	RM 228	130 bp	110 bp	100 bp, 130 bp
	RM 84	120 bp	100 bp	100 bp, 120 bp
	RM 279	150 bp	130 bp	130 bp, 150 bp
JRH 8	RM 234	130bp	150 bp	130bp, 150 bp
	RM 228	130 bp	110 bp	110 bp, 130 bp
	RM 279	150 bp	130 bp	130 bp, 150 bp
	RM 510	110 bp	130bp	110 bp, 130 bp
JRH 19	RM 279	160 bp	130 bp	130 bp, 160 bp
	RM 510	110 bp	120 bp	110 bp, 120 bp
	RM 228	130 bp	110 bp	110 bp, 130 bp
	RM 234	130 bp	150 bp	130 bp, 150 bp

informative markers (RM 510, RM 237, RM 455, RM 137, RM 428, RM 475, RM 202, RM 171, RM 234, RM 84, RM 228, RM 236, RM 85, RM 241, RM 488 and RM 42), which were used for fingerprinting of the hybrids. Eight primers amplified a maximum of two alleles whereas all other other monomorphic detected one allele across the genotypes (Fig. 1).

Testing genetic purity of hybrid seeds

Determining the genetic 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 test the genetic purity of hybrid JRH 5, the SSR markers RM 234, RM 228, RM 84, RM 279 and RM 237 were identified. For JRH 8 and JRH 19, markers RM 234, RM 228, RM 279 and RM 510 were identified for testing the genetic purity of hybrids (Fig.2). The summarized result of testing of genetic purity of all three hybrids presented in the table 5. The testing of genetic purity of hybrid using molecular markers shorten the time for testing by saving one full crop season and thus reducing the cost of hybrid seed. The present study revealed that comparative assessment of both data obtaned from the experiment involving GOT analysis and SSR markers was found *vis-à-vis* comparable. In the grow out test, purity analysis was conducted on the basis of morphological characters viz., plant height, days to 50% flowering, leaf colour, awning etc. The characters of the few individual that had shown the deviation from the standard set of characters were identified as off type. The results were confirmed by using the molecular

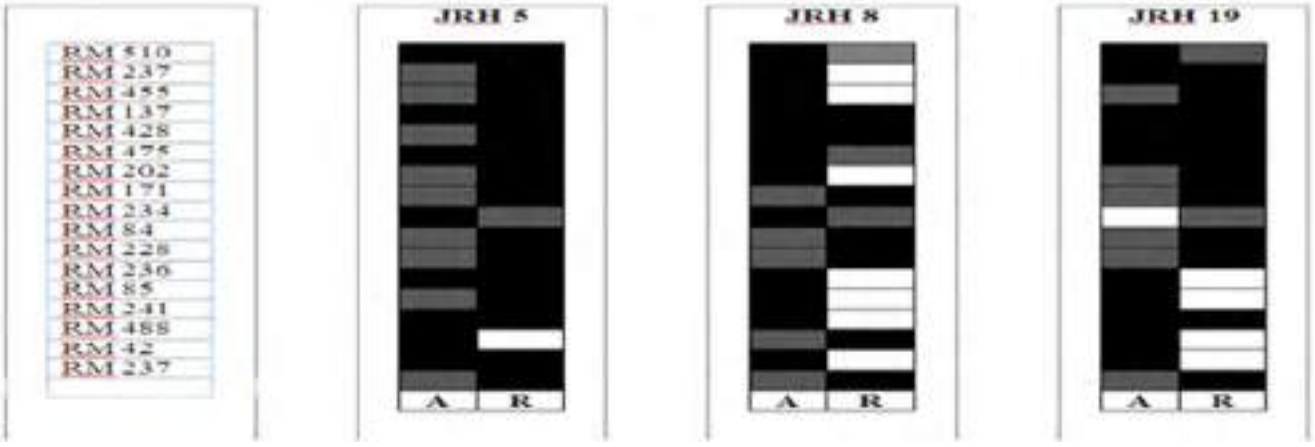

Fig. 1. Diagrammatic representation of DNA fingerprints of three rice hybrids based on 16 SSR markers. In each hybrid the column of A and R represent the allelic profile of CMS (A) and restorer (R) parent, respectively. The shades indicate different alleles amplified by the respective SSR markers in the hybrid

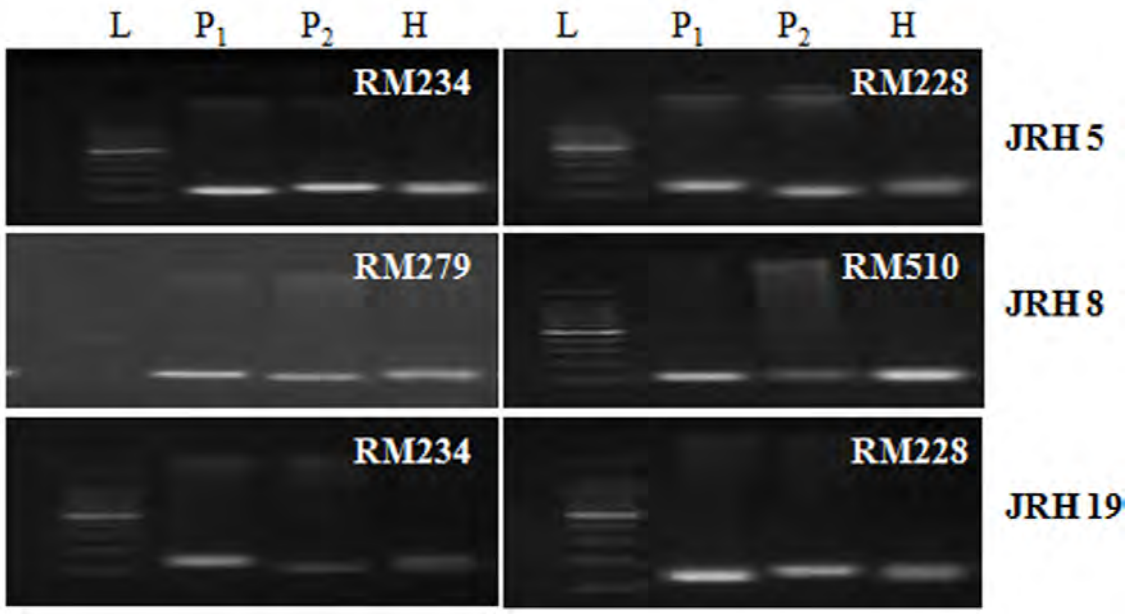

Fig. 2. Molecular profiling of rice hybrids JRH 5, JRH 8 and JRH 19. (L:100 bp ladder, P_1: A line, P_2=:R line, H: Hybrid)

markers and identified different unique markers for each hybrid.

REFERENCES

Hossain M D, Singh A K, Zaman F 2010. Genetic of fertility restoration of 'WA' based cytoplasmic male sterility system in rice (*Oryza sativa* L.) using indica/ japonica derivatives restorers. Science Asia, 36:94-99.

Jain S, Jain RK, McCouch S R 2004. Genetic analysis of Indian aromatic and quality rice (*Oryza sativa* L.) germplasm using panels of fluorescently-labeled microsatellite markers. Theoretical and Applied Genetics 109: 965-977.

Jena K K; Pandey SK 1999. DNA markers for purification of A and B lines for hybrid rice improvement. Hybrid Rice News Lett 2(1): 13-14.

Jones J W 1926. Hybrid vigor in rice. J. Am. Soc. Agron. 18:424-428.

McCouch S R, Chen X, Panaud O, Temnykh S, Xu Y, Cho Y G, Haung N, Ishii T, Blair M 1997. Microsatellite development, mapping and application in rice genetics and breeding. Plant Molecular Biology 35(1-2):89-99.

Nandakumar N, Singh A K, Sharma R K, Mohapatra T, Prabhu K V, Zaman F U 2004. Molecular fingerprinting of hybrids and assessment of genetic purity of hybrid seeds in rice using microsatellite markers. Euphytica 136:257-264.

Singh A K, Singh M K, Prasad S K, Sakarwar P 2014. Sequential herbicide application and nitrogen rate effects on weeds in direct seeded rice (*Oryza sativa* L.). The Ecoscan 8(3&4):249-252.

Yashitoal J, Thirumurugan T, Sundaram R M, Naseerullah M K, Ramesha M S, Sharma N P, Stone R V 2002. Assessment of genetic purity of rice hybrids using microsatellite and STS markers. Crop Sci. 42:1369-1373.