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Genetic purity assessment of indica rice hybrids through DNA fingerprinting and grow out test

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Abstract

Aim : Varietal identity and their purity assessment is essential to assert intellectual rights to breeder, helps seed testing and certification agencies to ensure pure seeds to the farmers, buyers, processors and consumers.

Methodology : In the present study, molecular characterization and genetic purity analysis of nine indica rice hybrids and respective 13 parental lines (22 genotypes) were done utilizing grow out test grow out test and micro-satellite markers based DNA fingerprinting approach. A total of 48 microsatellite (SSR and STMS) markers spanning over whole rice genome were screened.

Results : The statistical analysis of grow out test revealed that 88.25 per cent genetic purity was assessed in 3 hybrids tested. Twelve out of 48 microsatellite used were found polymorphic and amplified a total of 29 alleles among the genotypes with an average polymorphic information content value of 0.45. Out of 12 informative markers, nine were found polymorphic across the genotypes and produced unique fingerprints. Five STMS markers (RM154, RM164, RM234, RM258 and RM519) showed polymorphism over all the genotypes and were identified as referral marker for use in unambiguous identification and protection of nine hybrids. Nevertheless, marker RM 19 amplified allele was specific to the parental lines of CNRH 3 and APHR 2 (having common CMS line, IR62829). The results on genetic purity test through GOT had highly significant positive correlation ($r=0.96$) with molecular marker analysis.

Interpretation : Genotype specific STMS markers identified in the study and their close association with the ability of grow out test, emphasizes immense scope for molecular approaches to be an alternatives in unambiguous assessment of genetic purity of hybrids and their parents. The molecular markers based genetic purity assessment may serve as a probable substitute to grow out test, which is a cumbersome and time consuming task and would benefit the farmers and agencies involved in this venture, hybrid rice seed production and cultivation.

Introduction

Rice (*Oryza sativa*) is a staple food crop of global significance used as a primary food by more than half of the world's population (Singh *et al.*, 2013). It is a premier crop, in terms of its calorie contribution to human diet and monetary value of food production in developing world (Sasaki, 2005; Singh *et al.*, 2014). Globally, it occupies more than 146 million ha land which produces approximately 685 million tonnes of grain annually (RMM-USDA, 2015). It has great role in alleviating poverty and malnutrition, and reaffirming the need to focus world attention on its role in providing food security and eradicating poverty, especially in developing countries (IRRI, 2015). In India, rice occupies about 44 million ha of cultivatable area (~22% of total cropped area) with an annual production of 106.54 million tonnes, which contributes approximately 25% to agricultural GDP.

In Asian countries, most of the cultivable land suitable for rice production has already been exploited. The world population is expected to reach upto 8.2 billion with the global demand of approximate 533 million tonnes of milled rice by the end of year 2030 as compared to 477 million tons projected for 2015 (IRRI, 2015; RMM-USDA, 2015). In this scenario, hybrid rice technology is considered as a viable option to increase rice yield globally as it gives on an average about 20 - 25 % higher yield than the conventional high yielding varieties (Virmani, 1994; Kumar *et al.*, 2012). However, the unavailability of pure seed of hybrids at the affordable cost and lack of specific resistance to some major biotic and abiotic stresses are the major constraints in large scale adoption of the technology.

The ability to assess varietal identity and purity not only helps the plant breeder to assert breeders rights, but also benefits the variety and seed testing authorities, the certification agencies, farmers and buyers, processors and consumers. According to Mao *et al.* (1998) and Kumar *et al.* (2012) every one percent of impurity in hybrid seeds could reduce the yield upto 100 kg per ha⁻¹. The area of the most concern for the future hybrid rice programme is the long-term maintenance of the genetic purity of the hybrid, parental lines (A, B and R lines) and also maintenance of high level of genetic purity in hybrids is essential to exploit the moderate level of heterosis observed in crop (Yashitola *et al.*, 2002; Nandkumar *et al.*, 2004). Thus, there is a need to assess the genetic purity of hybrid seeds in quick succession so that seeds produced in dry season could reach the farmers for commercial cultivation in very next wet season.

The purity of hybrid seeds is conventionally assayed by conducting grow out test involving representative sample of the seeds to be marketed (Bora *et al.*, 2016). Grow-out test generally based on some highly heritable traits "descriptors" requires one full season to complete, hence, it is tedious and time taking and highly input extensive. Molecular markers, in contrast, offer an impartial means for precise molecular profiling of

varieties/hybrids and their purity assessment in short span of time (Rehem *et al.*, 2010). Microsatellite (SSR/ STMS) marker based high level of polymorphism or fingerprinting assays have now emerged as one of the new and rapid technology for cultivar/impurity identification to replace/ supplement the conventional grow-out test (Yashitola *et al.*, 2002; Yifru *et al.*, 2006; Lu *et al.*, 2009; Bora *et al.*, 2016). Cytoplasmic male sterile (CMS-A line), maintainer (B-line), restorer (R-line) lines and their hybrids have been screened by a number of microsatellite and sequence tagged site markers by several workers (Sundaram *et al.*, 2008; Jaikishan *et al.*, 2010; Kumar *et al.*, 2012, 2016; Dash *et al.*, 2016).

Therefore, keeping in view of the above facts, the objective of the present study was to estimate and identify the level of purity in seed lots of nine rice hybrids with their parental lines through microsatellite markers based DNA fingerprints and grow out test, and also to establish the basis for identification, authentication, protection and monitoring of seed purity for hybrids evaluated.

Materials and Methods

Experimental materials and practices : Geographically, the experimental site is situated at 29° N latitude and 79.3° E longitude at an altitude of 243.8 m above the mean sea level and in the foothills of Shivalik range of the Himalayas in narrow belt called Terai. Further, laboratory work was carried out in Rice Research Laboratory, Department of Genetics and Plant Breeding, G. B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India and Biotechnology Laboratory, Division of Crop Improvement, National Rice Research Institute, Cuttack, Odisha, India during 2013-14. The experimental materials used in this study comprised nine *indica* rice hybrids (Pant Sankar Dhan-1, Pant Sankar Dhan 3, Narendra Sankar Dhan 2, KRH-2, CORH-2, DRRH-1, Sahyadri, CNRH 3 and APHR-2) and thirteen parental lines including two CMS (A) lines with their maintainer (B) lines (IR 58025A and B, IR 62829A and B) and nine restorer (R) lines (UPRI 92-133R, UPRi 93-287R, NDR 30264R, MTU3026R, KMR-3R, C-20R, BR-827-35R, IR-407050R and Ajaya). These nine hybrids are well adapted to different climatic zones with good yield potential. The seeds were obtained from different institutions/agencies in the country.

Twenty-two lines were sown on well prepared seed-bed (Terai) to raise the seedlings during *Kharif* season (June to November) of 2010-2012. Twenty-one days old single seedlings were transplanted in randomized block design under three replications in separate plots (15 m²) of 3 meter length with 25 x 20 cm spacing. The fertilizers were applied @ 140 kg N, 80 kg P₂O₅ and 60 kg K₂O per hectare (as per recommendation of hybrid rice cultivation). Half of the N₂ and full dose of P₂O₅ and K₂O were used as basal dose. Remaining half of nitrogen was applied at the time of tillering. The experimental field was kept free from weeds by

adopting manual weeding and herbicide application. All the recommended cultural practices and plant protection measures were followed to raise the healthy crop. Laboratory work was carried out in Rice Research Laboratory, G. B. Pant University of Agriculture and Technology, Pantnagar, and Biotechnology Laboratory, National Rice Research Institute, Cuttack, during 2013-14.

Grow out test : Grow out test is used to determine the genuineness (true to the type) of variety/ hybrid and it is based on the morphological traits of variety/ hybrids which are highly suitable, easily categorized by naked eye and least influenced by the environment. For grow out test, thirteen morphological characters taken from DUS (distinguish uniformity stability) test viz., basal stem colour, leaf colour intensity, early flowering, late flowering, male sterility, stem length excluding panicles, altitude of blade in flag leaf, curvature of main axis of panicle, panicles awn, branching altitude of panicle, panicle exertion and time of maturity. These 13 observations were taken under investigation from eight selected lines viz., 3 hybrids (Pant Sankar Dhan 1, Pant Sankar Dhan 3 and Narendra Sankar Dhan 2) and one CMS line (UPRI 95-17 : A line), one maintainer line (UPRI 95-17 : B line) and 3 restorers (UPRI 92-133, UPRI 93-287 and NDR 30264 : R lines) of hybrids. The observations were recorded from randomly selected more than 200 plants in field plot and subjected to statistical analysis for correlation with molecular data.

Molecular marker based genetic purity analysis : Genomic DNA of each plant was isolated from fresh leaf (~ 100 mg each) samples (18 days old seedlings) using cetyl trimethyl ammonium bromide (CTAB) method following the protocol of Murray and Thompson (1980). DNA concentration was then analyzed by taking OD_{260/280} of each sample using Nanodrop followed by 0.8 % Agarose gel electrophoresis using uncut λ DNA as standard check and then dissolved in T10E1 buffer (pH 8.0) for storage and subsequent analysis.

Forty eight microsatellites (STMS and STS) markers were selected from the rice SSR linkage map (Temynkh *et al.* 2000, 2001; available online at <http://www.gramene.org>) based on the criteria: uniformity in distribution across the genome and high polymorphic information content. A total of 48 STMS markers were utilized of which only twelve (ten STMS and two STS, pTA248 and RM235 markers) were found polymorphic and utilized for molecular characterization of parents and respective hybrids. PCR reaction mixture (total volume of 25 μ l) contained 1.5 μ l (20 ng) genomic DNA sample, 0.5 μ l (40 μ mole l⁻¹) primers of forward and reverse sequences, 2.5 μ l 10 X PCR buffer, 0.5 μ l dNTPs mix (10 m mole l⁻¹), 1.5 μ l MgCl₂ (20 m mole l⁻¹), 0.3 μ l Taq DNA polymerase (5 U μ l⁻¹, MBI Fermentas Hanover, USA) and 17.7 μ l double distilled water. The PCR reaction was performed in Thermal Cycler (Mastercycler gradient, Eppendorf, Hamburg, Germany) as initial DNA denaturation at 94°C for 5 min followed by 35 - 39 cycles (94°C for 1 min, 55°C for 1 min, 72°C 1 min) and

final extension step at 72°C for 10 min. The annealing temperature was modified by redesigning (< GC%) the primers used for the study.

Amplified product was resolved in 3.0 % Agarose gel in 0.5 X TAE buffer and stained with Ethidium bromide (0.5 μ g ml⁻¹) for visualization. DNA amplified product were mixed with loading dye in 5:1 ratio before loading the samples in the Agarose gel for electrophoresis in 0.5X TAE buffer. The electrophoresis was carried out for about 3 hrs at 80 volts. The molecular weight marker (50 bp ladder and 100 bp ladder) were used to compare the molecular weights of amplified fragments. After electrophoresis, the gel was visualized under UV and photographed using Gel-Doc system (Alpha Innovation Technology Corporation, USA). Only clear-cut and unambiguous bands were scored and faint bands were omitted.

Statistical analysis : SSR and STS markers generated clear and unambiguous bands of various molecular weight sizes, were scored for absence (0) and presence (1) of the corresponding band among all the 22 genotypes (hybrids and parental lines) and a binary data matrix was prepared for further analysis using NTSYS-pc ver. 2.11W (Rohlf, 1997).

The SimQual programme was followed to calculate the Jaccard's similarity coefficients (Jaccard, 1908). The resulting similarity matrix was used for unweighted pair group method with arithmetic averages (UPGMA) based dendrogram (Sneath and Sokal, 1973) using the sequential agglomerative hierarchical nested cluster analysis (SAHN) module of NTSYSpc software.

The data generated from molecular markers banding pattern were also used for correlation coefficient analysis.

Results and Discussion

Molecular characterization of hybrids and their parental lines : Recent advancement in molecular techniques has opened the door through which specific molecular fingerprints of parental lines and hybrids that may serve the purpose, efficiently, can be developed (Yun *et al.*, 2005; Antonova *et al.*, 2006). In the beginning, PCR based RAPD marker was used for DNA fingerprinting of hybrids/ genotypes of rice (Wang *et al.*, 1994), but after the developments of microsatellite markers (Mc Couch *et al.*, 1997, 2002), STMS and STS, which are co-dominant in nature, has chromosomal specificity and can generate variety specific profiles with high fidelity and capable to differentiate between the homozygous and heterozygous genotypes (Yun *et al.*, 2005; Antonova *et al.*, 2006; Sundaram *et al.*, 2008; Vikas *et al.*, 2014). There are more than nineteen thousands of well distributed and mapped STMS and STS markers reported in the rice (Tamilkumar *et al.*, 2009; Lea *et al.*, 2010; Gramene, 2016), that have constituted a large group of markers for detecting polymorphism between parental lines of hybrids. A clear-cut identity and ensured genetic purity of seeds in hybrids/ varieties are important to protect the intellectual properties of developers (breeders) and also to the farmer's interest.

Objectively, 48 STMS markers were taken from Gramene database (www.gramene.org) covering 67% genome of rice were used to survey the polymorphism between the hybrids and parental lines. The high polymorphic information content (PIC) of STMS had promoted for application as molecular markers for DNA finger printing (Ashikawa *et al.*, 1999). Among 48 markers, 12 markers showed polymorphism (informative marker) which were further utilized for molecular characterization of parents/ hybrids and seed purity assessment of seed lots of respective hybrids and parental lines. The polymorphic survey in parental lines showed 108 heterozygous alleles with an average of nine alleles per marker. Amongst five markers, RM154, RM164, RM234, RM258 and RM519 were found to be most informative, amplifying the highest 13 heterotic alleles (hybrids and/or parental lines), and on contrary, marker RM 122 produced minimum number of alleles (2). Besides, RM19 showed highest PIC value (0.93) while RM154, RM519 and RM164 were shown least PIC (0.01) (Table 3). Out of 12 informative markers, 8 were (RM19, RM154, RM164, RM205, RM234, RM250, RM 258 and RM519) exhibited polymorphism and produced unique fingerprint throughout the parental lines and their hybrids. Five of these markers (RM154, RM164, RM234, RM258 and RM519) revealed heterozygosity over all hybrids and identified as reference markers for unambiguous identification (Figure 1). The STMS marker, RM 19 amplified alleles were specific to the parental lines of CNRH 3 and APHR 2. Similarly, two STMS marker RM 205, RM 250 and a STS marker pTA248 showed polymorphism in seven hybrids, Pant Sankar Dhan-1, Pant Sankar Dhan 3, Narendra Sankar Dhan 2, KRH-2, CORH-2, Sahyadri and DRRH-1. However, these markers did not show polymorphism in hybrids CNRH 3 and APHR 2 which might be due to the use of same CMS (IR 62829A) source in the development of these hybrids. Marker RM 203 was amplified in the parental lines (CMS lines, IR62829 and IR 58025, and respective maintainers) and failed to amplify in all the hybrids under study. These results are in close agreement with Yoshitola *et al.* (2002). Since STMS and STS markers cover whole genome, they show polymorphism in DNA banding pattern of different genotypes which reveal the variation and allelism (marker alleles) in larger quantity (Yashitola *et al.*, 2002; Nandkumar *et al.*, 2004; Sayyed *et al.*, 2009; Antonova *et al.*, 2006; Sundaram *et al.*, 2008; Jaikishan *et al.*, 2010; Pallavi *et al.*, 2011; Kumar *et al.*, 2012; Vikas *et al.*, 2014; Kumar *et al.*, 2016).

The variation in the PCR amplified product sizes in the parental lines revealed that marker RM154, RM234 and RM258 (Fig. 1.) had polymorphism in all nine hybrids and their parental lines amplified the band sizes of 140 to 270 bp, 130 to 270 bp and 140 to 230 bp, respectively. Moreover, RM164 showed an average band size of 200 bp and it ranged upto 140 to 240 bp and RM519 showed band size of 130 to 200 bp over all the parental lines screened. RM250 and RM205 produced polymorphic pattern in parental lines of seven hybrids and produced band ranging from 120 to 200 bp. However, RM 19 showed polymorphic pattern only in parental lines of hybrids CNRH 3 and

APHR 2 (both have common CMS source *i.e.*, IR 62829A) and produced bands in the range of 150 - 200 bp. A STS marker pTA 248 showed polymorphism for all the hybrids and parental lines screened. The RM235 could not distinguish heterozygosity between parental lines of any hybrid in the PCR amplification. The markers RM 250 and pTA248 were found to be polymorphic for all the parental lines of hybrids except for CNRH 3 and APHR 2 (involved CMS, IR62829A as female), which suggests to use these markers for testing the genetic purity of hybrids developed utilizing CMS IR 58025A as the female parent. Though the STMS marker RM19 produced polymorphic pattern in parental lines of hybrids CNRH 3 and APHR 2, but failed to amplify in remaining seven hybrids in one PCR condition. There is possibility to obtain polymorphism between parental lines of hybrids with some modification in PCR conditions (Nandkumar *et al.*, 2004; Jaikishan *et al.*, 2010; Lea *et al.*, 2010). It indicated large variation in the frequency of heterozygosity in all hybrids tested, which ranged from 6 % to 70 % for STMS loci and 0 to 50 % for STS loci. Of the 12 informative markers used, 5 markers were identified to detect the polymorphism in all the parental lines and nine hybrids. Thus, it seems that these 5 markers can be widely employed for identification of these hybrids and their parental lines.

DNA profiling and genetic purity assessment : Genetic adulteration in hybrid seeds caused drastic reduction in heterosis expression was studied by molecular markers in *indica* rice hybrids (Mao *et al.*, 1998; Yun *et al.*, 2005). In hybrid breeding programme, maintenance of genetic purity in parental lines is most important feature for the development of pure/ true hybrid seeds. Though grow out test is one of the conventional and standard techniques commonly used for the assessment of genetic purity of seeds, it is not that much popular due to its limitations, especially time consuming process. The recent molecular technologies for genetic purity assessment are rapid, robust and reliable (Yashitola *et al.*, 2002). The assessments of heterozygosity of parental lines and hybrids were undertaken using five hybrid specific STMS markers *viz.*, RM154, RM164, RM234, RM258 and RM519, which were identified through polymorphism survey. Fifty seeds from each parental lines were subjected to draw the fingerprinting based on STMS markers. It was found that only one parental line IR 62829A showed one heterozygote for all the markers tested, while their B-line of same CMS and restorer lines did not detect any heterozygote bands using all five markers. Further analysis showing heterozygote bands confirm the impurities of 4% in seed lots of IR 62829A CMS lines.

Assessment of genetic purity of hybrids (10 hybrids, included one more hybrid seed lot of Pant Sankar Dhan 1 produced and marketed by M/S Syngenta India Ltd.) by application of molecular markers (five STMS) were carried out on 50 randomly selected seeds from seed lot of each hybrid. Genetic purity analysis indicated the maximum number of off-type plants in hybrid seed lot of Narendra Sankar Dhan 2 (6 plants) (Fig. 2) followed by CNRH 3 (4 plants) and Pant Sankar

Table 1 : Description of 9 indica rice hybrids used in genetic purity assessment with their pedigree and features

Name of hybrid	Parentage and year of release	Source institution	Specific features of indica rice hybrids
Pant Sankar Dhan-1	IR 58025A x UPRI 93-133R 1997	G. B. Pant University of Agriculture & Technology, Pantnagar, Uttarakhand -do-	Short duration (115-120 days) with long slender grains, moderate resistant to BLB, brown spot and blast, free to false smut and kernel bunt under field conditions, yield: 68 qha ⁻¹
Pant Sankar Dhan -3	IR 58025A x UPRI 92-287R 2004		Mid early (125-130 days), Semi tall (115 cm), sturdy culm Non-lodging, fertilizer responsive Long slender grains with mild aroma, intermediate amylose (20.0%) and high ASV (6.5), moderately resistant to bacterial blight, stem borer, BPH/ WBPH, leaf folder and yield: 62 qha ⁻¹ .
Narendra Sankar Dhan -2	IR 58025A x NDR-30264 1998	N. D. University of Agriculture & Technology, Faizabad, U.P.	Mid early (125-130 days), semi tall (114 cm), non-lodging, tolerant to shattering, responsive to fertilizer, long slender grains with high milling (70%), low HRR (46%), yield: 62 qha ⁻¹
KRH-2	IR 58025A x KMR-3 1996	VC Farm Mandya, UAS, Bangalore, Karnataka	Medium duration (130-135 days) with long bold grains, yield 74.0 q/ha, released for state of AP, Karnataka, T. N., Tripura, Maharashtra, Haryana, Uttarakhand, Odisha, W.B. and Rajasthan.
CORH-2	IR 58025A x C20R 1998	Tamil Nadu Agriculture University, Coimbatore, Tamil Nadu	Medium duration (120-125 days), Semi dwarf (102 cm), with long slender grains, white, tolerant to Leaf blight, bacterial streak and other diseases; yield: 70.0 - 72.0 q/ha
DRRH-1	IR 58025A x IR 40750 1996	Indian Institute of Rice Research (IIRR), DRR Rajendranagar, Hyderabad	Medium duration (125-130 days) with long slender grains, released for state of Andhra Pradesh, yield: 73 q/ha, released for the state of Andhra Pradesh, Odisha and Karnataka.
Sahyadri	IR 58025A x BR 827-35 1998	Regional Agricultural Research Station, Karjat	Medium duration (125-130 days) with long slender grains, yield: 66 to 68 q/ha and released for state of Maharashtra
CNRH-3	IR62829A x Ajaya 1995	Rice Research Station, Chinsurah, West Bengal	Medium duration (125-130 days) with medium slender grains, yield: 70 to 75 q/ha and released for the state of W.B.
APHR-2	IR62829A x MTU 9992 1994	Andra Pradesh Rice Research Institute, Maruteru, Andhra Pradesh	Medium duration (120-125 days), long slender grains hybrid, semi dwarf (100 - 105 cm), grains - long slender, white, AWA; yield: 75 to 76 q/ha, released for state of Andhra Pradesh

Table 2 : List of microsatellites/STS markers used in screening of hybrid and parental lines

S.No.	Markers	Chromosome number	Motif & No. of repeat	Size range (bp)
1	RM12233	1	(AGG)8	83
2	RM173	1	{{(GT)3T2AGGGACA}2	173
3	RM220	1	(CT)17	127
4	RM10746	1	(AG)10	173
5	RM203	2	(AT)21	203
6	RM154 (OSR11)	2	(GA)21	183
7	RM250 (RM14024)	2	(CT)17	153
8	RM263	2	(CT)34	199
9	RM 15981	3	(AG)46	259
10	RM 14811	3	(AG)19	332
11	RM1510	3	(CCG)7	183
12	RM15621	3	(AG)12	282
13	RM19	4	(ATC)10	226
14	RM16284	4	(AG)15	292
15	RM16336	4	(AGAT)5	569
16	RM16762	4	(CCG)7	100
17	RM 18336	5	(AT)34	256
18	RM18004	5	(AAG)15	189
19	RM164 (RM18617)	5	(GT)16	246
20	RM122	5	(GA)11	227
21	RM 20522	6	(AG)23	122
22	RM 19456	6	(AAT)20	183
23	RM19546	6	(AAG)7	394
24	RM20512	6	(AG)14	236
25	RM 21395	7	(AG)13	90
26	RM 21427	7	(AAG)8	189
27	RM 20810	7	(AT)34	197
28	RM234 (RM21972)	7	(CT)25	156
29	RM23107	8	(AG)14	96
30	RM22230	8	(AG)34	291
31	RM23528	8	(AGG)8	104
32	RM 22914	8	(AG)29	0
33	RM24412	9	(AG)11	290
34	RM2456 0	9	(GCG)7	420
35	RM205	9	(CT)25	122
36	RM201	9	(CT)17	158
37	RM228	10	(CA)6(GA)36	154
38	RM258 (RM25600)	10	(GA)21	148
39	RM216	10	(CT)18	146
40	RM171	10	(GATG)5	328
41	RM27235	11	(AC)12	266
42	RM224	11	(AAG)7	20
43	pTA 248	11	-	750
44	RM206	11	(CT)21	147
45	RM27644	12	(AG)11	278
46	RM519 (RM28302)	12	(AAG)8	122
47	RG235	12	(CT)24	124
48	RM247	12	(CT)34	199

Dhan 1 (2 plants). Hybrids DRRH 1, COHR 2 and APHR 2 showed 3 off-type plants each in their seed lots, while seed lots of hybrid Pant Sankar Dhan 3 and Sahyadri indicated much greater genetic purity with only single off-type plant.

Genetic similarity/dissimilarity among hybrids and parental lines : Genetic diversity among parents is key determinant to

predict the level of heterosis in hybrid well advance. Purposely, a total of 12 informative microsatellite markers revealed varying degree of genetic relatedness among nine *indica* rice hybrids and their 13 parental lines (22 genotypes) were taken to assess the genetic diversity among hybrids/lines. Since Jaccard's similarity coefficient ranged from 0.77 to 0.99, a wide variation was observed between and among 22 genotypes due to diversified

Table 3: Total number of off-type plants observed at different stages of plant growth to plant maturity in two years (pooled over year)

Traits	Varieties /Genotypes								Total
	UPRI 95-17A	UPRI 95-17B	UPRI 92-133R	UPRI 93-287R	NDR 30264R	Pant Sankar Dhan-1	Pant Sankar Dhan-3	Narendra Sankar Dhan-2	
Basal stem colour	R ₁	0	3	1	0	0	2	0	6
	R ₂	0	2	2	0	0	2	0	6
Leaf: intensity of green colour	R ₁	1	0	0	0	1	0	3	5
	R ₂	1	0	0	0	0	0	1	2
Early flowering	R ₁	0	2	0	0	2	1	0	7
	R ₂	2	0	1	0	3	0	0	7
Late flowering	R ₁	1	1	0	0	1	0	1	7
	R ₂	0	1	0	0	0	0	0	1
Male sterility	R ₁	2	0	0	1	0	0	0	3
	R ₂	1	0	0	0	0	0	0	1
Stem: length (excluding panicle)	R ₁	0	0	0	0	0	0	2	2
	R ₂	0	0	0	0	0	0	0	0
Flag leaf: attitude of blade	R ₁	0	0	0	0	0	1	0	1
	R ₂	0	0	0	0	0	3	0	3
Panicle :curvature of main axis	R ₁	0	1	4	0	1	0	1	7
	R ₂	0	2	0	0	2	0	2	6
Panicle: awns	R ₁	0	2	0	0	0	0	2	4
	R ₂	0	2	0	0	0	0	3	5
Panicle: attitude of branching	R ₁	0	0	0	0	0	1	0	1
	R ₂	0	0	0	0	0	1	0	1
Panicle exsertion	R ₁	1	0	0	0	0	0	0	1
	R ₂	2	0	0	0	0	0	0	2
Plant height	R ₁	1	1	0	1	0	2	0	6
	R ₂	0	0	0	2	0	1	0	3
Time of maturity	R ₁	1	2	0	0	0	0	1	4
	R ₂	0	1	0	0	0	0	2	3
Total		13	15	10	7	10	10	11	94

R1 and R2 stands for replication one and two, respectively

morphology and variation among the rice genotypes (Fig. 3). These informative markers were grouped nine hybrids in two clusters viz., first group had 7 hybrids and second group had 2 hybrids (CNHR3 and APHR2) (Fig. 4). However, 13 parental lines were grouped in two main clusters and five sub-categories based on genetic similarities. High genetic similarity coefficient (0.99) was found between five pairs of genotypes viz., restorer line NDR30264R and IR40705R, sterile and maintainer line IR62829A and IR62829B, hybrids CNHR3 and APHR2, Pant Shankar Dhan1 and KRH2. Whereas, least genetic similarity was observed 0.77 between 10 pairs of genotypes viz., MTU9992 and IR58025B, Ajaya and NDR30264R, APHR2 and KRH2, CNHR3 and COHR2, Ajaya and KMR3 etc.

The UPGMA Dendrogram of 22 rice genotypes including nine hybrids were grouped into two main clusters consisting of 18 and 4 genotypes, respectively (Fig. 3). Cluster I was further divided into five sub-clusters i.e., sub-cluster Ia (two genotypes: IR62829A and IR62829B), Ib (seven genotypes/ hybrids: PSD1, PSD3, NSD2, KRH2, COHR2, DRRH1 and Sahyadi), Ic (UPRI93-133R, UPRI93-287R, KMR3, C20R and BR827-35) and Id consisted of two genotypes IR58025A and IR58025B. However, cluster II was also divided into two sub-clusters, sub-cluster IIa and IIb, each consisted only two genotypes viz., Ajaya and MTU9992; CNHR3 and APHR2, respectively. Clustering pattern of parental line showed substantial genetic distance between parents which in fact led the level of heterosis in studied

hybrids. Similar finding was observed in recent past by Rajendran *et al.* (2012) who reported two major clusters of the maintainer and restorer groups with sub-clusters in each group and overall genetic similarity index value ranging from 0.69 to 0.87 with mean value of 0.78.

Grow out test : The most important quality characteristic of a hybrid is its reliability or purity. In scientific seed production programme, inspite of intense care to maintain the parental lines as well as hybrids purity some extent of impurity may occur due to improper flowering isolation and varietal admixtures. Grow out test has been used as one of the standard conventional approach to determine the true-to-type seeds of parental lines and their hybrids (Sonti *et al.*, 2003; Garg *et al.*, 2006). In this study, genetic purity of 8 genotypes (3 hybrids, Pant Sankar Dhan1, Pant Sankar Dhan 3 and Narendra Sankar Dhan 2; and 5 parental lines) from 4 seed lots of hybrids was assessed through conventional approach, grow out test. A total of 94 off-type/mixture plants were found under field evaluation. Highest number of off-type plants was found in hybrids Narendra Sankar Dhan-2 (18 plants) followed by Pant Sankar Dhan 3 (11 plants) and lowest in hybrid Pant Sankar Dhan 1 (10 plants) (Table 3).

In parental lines, UPRI 95-17B exhibited maximum number of off-type plants (15 plants) followed by UPRI 95-17A (13 plants) and UPRI 92-133R and NDR 30264R (10 plants in each).

Fig. 1: The molecular profiles obtained by STMS marker RM 258 of 9 *indica* rice hybrids with respective parents (A and R lines). M= 50bp ladder, A = CMS line (A line), H = hybrid (F₁ hybrids), R = restorer line (R line)

Fig. 2: STMS markers (RM154, RM164, RM234 and RM519) based genetic purity (mixture seed) assessment of hybrid Narendra Sankar Dhan-2 (NSD-2) with their parental line. Band size varies approximately 150 to 260 bp. Lane 1= IR 58025A, Lane 2= ND 30264R and lane 3-51 represents individual F₁ hybrid plants taken by random sampling from hybrid seed lot of NSD 2. Arrow represents in sample 14, 30, 31, 32 and 46 showed mixture/ off-type plants in hybrids

Fig. 3 : Dendrogram of 22 rice genotypes including 9 hybrids based on Jaccard's similarity coefficients based on 12 informative STMS markers

The lowest number of off-type plants were recorded in UPRI 93-287R (7 plants). Morphological characterization of genotypes showed maximum number of off-type plants with reference to certain traits i.e., early flowering, panicle curvature of main axis, basal stem colour and plant height. The off-type plant were observed for early flowering, followed by panicle curvature of main axis, basal stem colour and plant height is 14, 13, 12 and 9 plants, respectively. However, the lowest numbers of off-type plants were observed in case of stem length (excluding panicle) and attitude of panicle branching with two plants. In grow out test, the tested parental lines and hybrids indicated the maximum percentage of off-type plants in hybrid Narendra Sankar Dhan-2 (19%) and lowest in UPRI 93-287R (7.44%). Further, off-type plants were differentiated on the basis of their traits where highest off-type plants were observed with respect to early flowering, late flowering and panicle curvature of main axis (7.44% for each) and minimum of 1.06 % for the stem length (excluding panicle). Thus, statistical analysis of grow out test revealed 88.25 % genetic purity in three hybrids tested. Though, grow out test is used as a standard conventional approach in differentiation of hybrids, however, there are some limitations like more time consuming and inconvenient process of screening of genotypes (Kumar *et al.*, 2012).

Efficacy of molecular marker and grow-out test : Four seed lots of three hybrids Pant Sankar Dhan 1, Pant Sankar Dhan 3 and Narendra Sankar Dhan 2 were utilized for correlation coefficient analysis between off-type plants in seed lots of hybrids through grow out test and molecular marker based DNA fingerprinting analysis. The results showed highly significant positive association ($r = 0.96$) between grow out test and molecular marker based fingerprinting for genetic purity assessment of hybrids and parental lines (Kumar *et al.*, 2012; Vishwanath *et al.*, 2012). Thus, the results of molecular markers based fingerprinting in the laboratory can be used as a substitute for grow-out test. Markers identified in the present study can be utilized for genetic purity testing of the hybrids. The STMS marker information developed through this study will certainly be of enormous help for hybrid rice seed industry to select appropriate marker combinations and assess genetic purity of the parental lines of hybrids.

Hence, genotype specific STMS markers identified in the study and their close association with the ability of "grow-out test", emphasizes immense scope for molecular approaches to be an alternatives in unambiguous assessment of genetic purity of

hybrids and their parents seed. The markers identified may be useful as reference markers in future breeding programme and seed production programme for identification and protection of these nine hybrids from mixture of other variety seeds. The molecular markers based genetic purity assessment can be an effective, rapid and robust technique for the assessment of genetic purity of hybrids and would have great benefit to the farmers and agencies involved in this venture, hybrid rice seed production and cultivation.

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