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ANALYSIS OF GENETIC PURITY OF RICE HYBRIDS AND THEIR PARENTAL LINES USING MICROSATELLITE MARKERS

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Abstract

The present experiment was conducted with a view to identifying distinct microsatellite markers to assess fingerprinting of rice (*Oryza sativa* L.) hybrids, assessing variation within parental lines and testing the genetic purity of hybrid seed. Seventeen microsatellite markers were used for fingerprinting 15 rice hybrids and their parental lines. A total of 272 alleles were obtained from 17 microsatellite primer pairs with an average of 16 alleles per primer pair. The number of alleles amplified from each primer pair ranged from 10 to 23. Out of 17, seven microsatellite markers together differentiated all the 15 hybrids and the parental lines including two inbred variety. The microsatellite marker, RM72 distinguished parental lines of HB09 and Heera2, RM584 for Shakti and LP70, RM248 for BRRI hybrid dhan1, LP106, inbreds BRRI dhan28 and BRRI dhan29, RM211 for BRRI hybrid dhan2, ACI93024, RM219 for BRRI hybrid dhan3, ACI1 and LP108, RM20 for Gold and Tia and RM18 for SL08 to differentiate parental lines with its respective hybrids. RM229, the microsatellite marker distinguished CMS lines and their corresponding restorer lines for most the hybrids.

Introduction

With the development of seed industry, introduction of hybrid rice varieties increased rapidly in the market. Use of hybrid rice seed has increased rapidly at farmer's level. Farmers are not aware of the quality of their purchased hybrid rice seed. There is no arrangement of testing the genetic identities of hybrid rice seed available in the market at the government level. So, there are chances of deprivation of the farmers for buying low quality seed.

As demands for feeding the rising world population, need for crop plants yield improvement is being more apparent. Rice is the main food of millions of people in the world. Hybrid rice possesses a yield advantage of 10 - 20% over the best inbred varieties (Virmani *et al.* 2003). Its commercial production has recently been attempted in other countries, following its success in China (Nandakumr *et al.* 2004). The strictly self pollinating nature of rice necessitates the exploitation of a male sterility. Production of rice hybrids using a cytoplasmic male sterility (CMS) system is based on and the fertility restoration system (Virmani *et al.* 2003), hence involves 3 lines of male sterile (A), maintainer (B) and restorer (R) line.

The fingerprinting of rice hybrids and identification of their genetic relationships are very important for plant improvement, variety registration system, DUS (distinctness, uniformity and stability) testing, seed purity testing and the protection of plant variety and breeders' rights. Accordingly, clear-cut identification of elite crop varieties and hybrids is essential for protection.

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and prevention of unauthorized commercial use (Nandakumr *et al.* 2004). On the other hand, purity of hybrid seeds supplied to farmers must surpass 96% (Ichii *et al.* 2003). Conventional characterization of hybrids based on specific morphological and agronomic data is time-consuming, restricted to a few characteristics, influenced by environmental condition and inefficient. Molecular marker assisted identification with high power of genetic resolutions has emerged as a robust technique for cultivar fingerprinting, identity profiling, estimating and comparing genetic similarity, and variety protection. Several types of molecular marker *viz.*, allozymes (Devanand *et al.* 1999), RAPD (Wang and Lu 2006, Ichii *et al.* 2003), SSR (Yashitola *et al.* 2002, Nandakumar *et al.* 2004) and STS (Yashitola *et al.* 2002) have been used in this term.

Thus, there is the need to identify, evaluate and characterize the available rice genotypes at both morphological and molecular levels to diversify the genetic base of improved rice varieties (Ogunbayo *et al.* 2005). Keeping the above aspects in view, the present study was carried out to identify microsatellite markers capable of distinguishing rice hybrids and their parental lines and to protect import of same variety with different name.

Materials and Methods

The field experiment was conducted at Seed Science and Technology laboratory and experimental field of Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Gazipur, and Biotechnology laboratory of Bangladesh Rice Research Institute (BRRI), Gazipur during the period of 2010 to 2012 Boro seasons.

The materials for this study comprised of 15 hybrids along with their CMS restorer lines and two high yielding varieties of rice. The pure seeds of all these genotypes were collected from different leading private seed companies of Bangladesh, hybrid rice division and genetic resource and seed (GRS) division of Bangladesh Rice Research Institute (BRRI), Joydevpur, Gazipur. List of the materials are presented in Table 1.

Seeds of all genotypes were grown in screen house condition at Department of Genetics and Plant Breeding, Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Salna, Gazipur. Five grams of germinated seed from each genotype was sown in the pot. The seedlings were allowed to grow for 21 days in order to get enough leaf material for extraction of DNA.

In total 47 genotypes comprise 15 hybrids and its corresponding CMS and restorer lines along with two inbred check variety. Collected leaf samples were evaluated at Biotechnology Laboratory of BRRI during Boro season 2011-12.

Forty microsatellite or simple sequence repeat (SSR) markers were used for selection (McCouch *et al*. 2002, IRGSP 2005). The seventeen SSR markers (RM13, RM18, RM20, RM31, RM72, RM119, RM128, RM211, RM219, RM228, RM229, RM231, RM248, RM280, RM314, RM448, RM584 distributed in the 12 chromosomes) with clear amplifications were selected for analysis of 47 genotypes. 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 spectro-photometer. DNA was diluted in TE buffer for PCR analysis. DNA amplification was carried out in a 15 ul reaction volume containing 0.2 uM of each primer, 200 RM of deoxyribonucleotides, 50 mM Ka, 10 mM Tris HC1 (pH 8.3), 1.5 mM $MgC1_2$, 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 hrs. The gels were next stained in ethidium bromide for 30 min, de-stained for 15 - 30 min and then observed under a UV transilluminator. Size for each amplified allele was measured in base pair using Alpha-Ease FC 5.0 software (Alpha Innotech, USA). Polymorphism information content (PIC) values were determined using Power Marker version 3.25 (Liu and Muse 2005). The allele frequency data from Power Marker were used to export in binary format (allele presence = 1 and allele absence = 0) for analysis with NTSYS-pc version 2.1 (Rohlf 2002). The resultant similarity matrix was employed to construct dendrograms using Sequential Agglomerative Hierarchical Nesting (SAHN) based Unweighted Pair Group Method with Arithmetic means (UPGMA).

Table 1. List of the hybrids along with their parental lines and sources.

Sl. No.	Hybrids	CMS lines	Restorer lines	Company/Institute	Source country
01.	HB 09F_1	HB 09A	HB 09R	BRAC	China
02.	Shakti F_1	Shakti A	Shakti R	BRAC	China
03.	BRRI hybrid dhan1	IR58025A	BR827R	BRRI	IRRI/BRRI
04.	BRRI hybrid dhan2	BRRI10A	BRRI10R	BRRI	Bangladesh
05.	BRRI hybrid dhan3	BRRI11A	BRRI15R	BRRI	Bangladesh
06.	Heera2 F_1	Heera2A	Heera2R	Supreme seed	China
07.	Heera5F_1	Heera5A	Heera5R	Supreme seed	China
08.	ACI1F_1	ACI1A	ACI1R	ACI Ltd.	China
09.	ACI93024F_1	ACI93024A	ACI93024R	ACI Ltd.	India
10.	LP70F_1	LP70A	LP70R	Abtab Bhamukhi Farm Ltd.	China
11	LP106F_1	LP106A	LP106R	Abtab Bhamukhi Farm Ltd.	China
12	LP108F_1	LP108A	LP108R	Abtab Bhamukhi Farm Ltd.	China
13	Gold F_1	Gold A	Gold R	Lal Teer seed Ltd.	China
14.	Tia F_1	Tia A	Tia R	Lal Teer seed Ltd.	China
15.	SL08F_1	SL08A	SL08R	BADC	Philippines
16.	BRRI dhan28 (Check)				Bangladesh
17.	BRRI dhan29 (Check)				Bangladesh

BRRI = Bangladesh Rice Research Institute, IRRI = International Rice Research Institute, BADC = Bangladesh Agricultural Development Corporation.

Results and Discussion

Fifteen hybrids and their parental A and R lines and two HYV were assessed for genetic variability using DNA fingerprinting technology. Forty microsatellite markers were tested initially from which 17 primers were selected as it exhibited polymorphism. The 17 polymorphism markers (RM13, RM18, RM20, RM31, RM72, RM119, RM128, RM211, RM219, RM228, RM229, RM231, RM248, RM280, RM314, RM448, RM584) 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 272 alleles were obtained using 17 SSR primer pairs with an average of 16 alleles per primer. The number of alleles amplified for each primer pair ranged from 10 to 23. The Polymorphic Information Content (PIC) for these primers ranged from 0.86 to 0.95 (Table 2). The marker RM548 generated a maximum number of 23 alleles, while RM

229 exhibited 22 and RM31 and RM 228 exhibited 20 polymorphic alleles. The average number of alleles per primer was 16. The primers showed an average PIC value of 0.90 which confirms the fact that, the SSR primers used in this study were highly informative. Markers with PIC values of 0.5 or higher are highly informative for genetic studies and are extremely useful in distinguishing the polymorphism rate of a marker at a specific locus (DeWoody *et al*. 1995). PIC values revealed that RM584, RM314 and RM229 are the best markers for distinguishing 15 hybrids including their parental A and R lines and two inbred.

Table 2. Data on the number of alleles, allele size range, major frequency allele and PIC for 17 microsatellite markers.

Marker	Chr. No	Position (Mbp)	Allele No.	Size range	Major allele	Major Allele Frequency	PIC
RM 128	1	31.06	11	161-173	166	0.2234	0.86
RM211	2	4.16	15	159-189	184	0.1383	0.90
RM231	3	0.45	12	182-194	187	0.1702	0.88
RM119	4	21.22	16	145-169	156	0.1383	0.91
RM280	4	34.95	15	146-172	149,156,158	0.1277	0.91
RM13	5	8.87	14	166-191	179	0.1915	0.89
RM31	5	28.59	20	151-181	164	0.0957	0.93
RM 314	6	9.71	10	139-151	144	0.2553	0.95
RM584	6	3.41	23	128-164	132	0.0638	0.83
RM18	7	26.65	15	109-127	122	0.2234	0.88
RM 248	7	29.33	13	115-142	132	0.2021	0.88
RM 72	8	17.60	16	133-168	155	0.1277	0.91
RM 219	9	3.38	17	83-199	102	0.1596	0.91
RM 228	10	21.98	20	161-184	171,172	0.1277	0.93
RM 229	11	18.37	22	189-294	209,217	0.0957	0.94
RM 20	12	0.97	12	115-132	119,123,125	0.1277	0.89
RM 463	12	22.09	18	99-125	113	0.1277	0.93
Mean			16			0.1527	0.90

The analysis using 17 evenly distributed hyper polymorphic SSR markers allowed to identify several markers, which exhibited amplification of alleles ‘specific’ or ‘unique’ to a particular parental line. The fingerprints of CMS lines and the restorer lines were also distinct, except two inbred BRRI dhan28 and BRRI dhan29, which had identical profiles with respect to most of the informative markers used in the study. DNA profile of markers RM314 and RM229 for all 47 genotypes of hybrids and their parental lines including BRRI dhan28 and BRRI dhan29 are shown in Figs 1 and 2.

Similar results were observed by fingerprinting and diversity studies, having 1 - 8 alleles with an average of 4.58 alleles for various classes of microsatellite (Siwach *et al.* 2004) and also 3 to 9 alleles, with an average of 4.53 alleles per locus for 30 microsatellite markers (Hossain *et al*. 2007). In the microsatellites, amplified polymorphic alleles in the parents (CMS and restorer) and these alleles were amplified co-dominantly in the hybrids (Figs 1 and 2). Similar findings also reported by Sonti *et al.* (2005) when they working with DNA markers to assess genetic purity of rice hybrids. The microsatellite marker, RM72 helped to differentiate parental lines of HB09 and Heera2 likewise RM584 for Shakti and LP70, RM248 for BRRI hybrid dhan1, LP106, inbreds

BRRI dhan28 and BRRI dhan29, RM211 for BRRI hybrid dhan2, ACI93024, RM219 for BRRI hybrid dhan3, ACI1 and LP108, RM20 for Gold and Tia and RM18 for SL08 used to differentiate parental lines with its respective hybrids. RM229 distinguished CMS lines and restorer lines of most hybrids (Fig. 2).

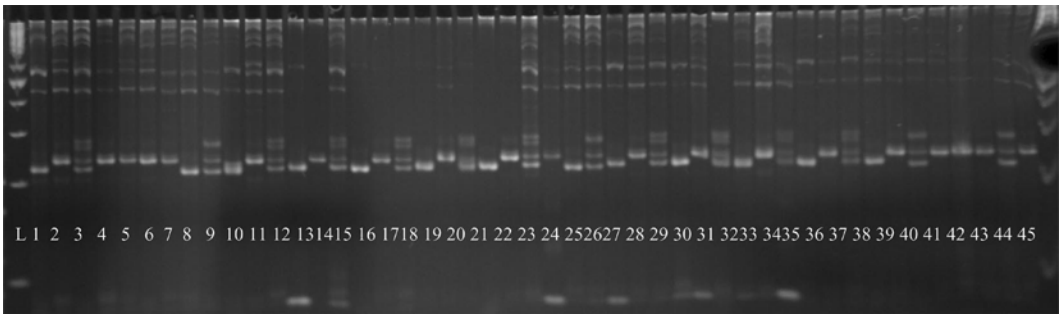

Fig. 1. DNA profile of 47 hybrid genotypes with RM314.

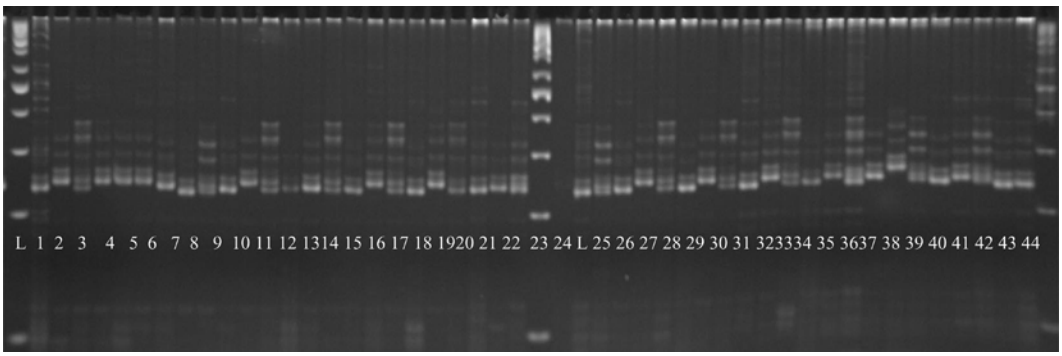

Fig. 2. DNA profile of 47 hybrid genotypes with RM229.

Legend: 1=HB09A, 2=HB09R, 3=HB09F_1, 4=ShaktiA, 5=ShaktiR, 6=ShaktiF_1, 7=IR58025A, 8=BR827R, 9=BRRI hybrid dhan1, 10=BRRI10A, 11=BRRI10R, 12=BRRI hybrid dhan2, 13=BRRI11A, 14=BRRI15R, 15=BRRI hybrid dhan3, 16=Heera2A, 17=Heera2R, 18=Heera2F_1, 19=Heera5A, 20=Heera5R, 21=Heera5F_1, 22=ACI1A, 23=ACI1R, 24=ACI1F_1, 25=ACI93024A, 26=ACI-93024R, 27=ACI93024F_1, 28=LP70A, 29=LP70R, 30=LP-70F_1, 31=LP106A, 32=LP106R, 33=LP106F_1, 34=LP-108A, 35=LP108R, 36=LP108F_1, 37=GoldA, 38=GoldR, 39=Gold F_1, 40=TiaA, 41=TiaR, 42=TiaF_1, 43=SL08A, 44=SL08R, 45=SL08F_1, 46=BRRI dhan28 (Check), 47=BRRI dhan29 (Check), L=ladder.

UPGMA based dendogram obtained from the binary data deduced from the DNA profiles of the analyzed samples. A total of 16 distinct groups resulted out of analysis of pooled SSR marker data at a cut-off similarity coefficient 0.32 (Fig. 3). UPGMA clustering system of the 15 hybrids and their parental lines revealed that they have very strong parental linkage (Haque *et al*. 2002). This fingerprinting data help identifying the genotypes very easily and the information generated from the study could be used in further molecular characterization with other hybrid genotypes. In case of hybrids, F_1 seeds are commercially grown by farmers, which make it necessary to use the fresh seed each year. Although the hybrids are costly the farmers grow hybrids because of higher yield, and 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.

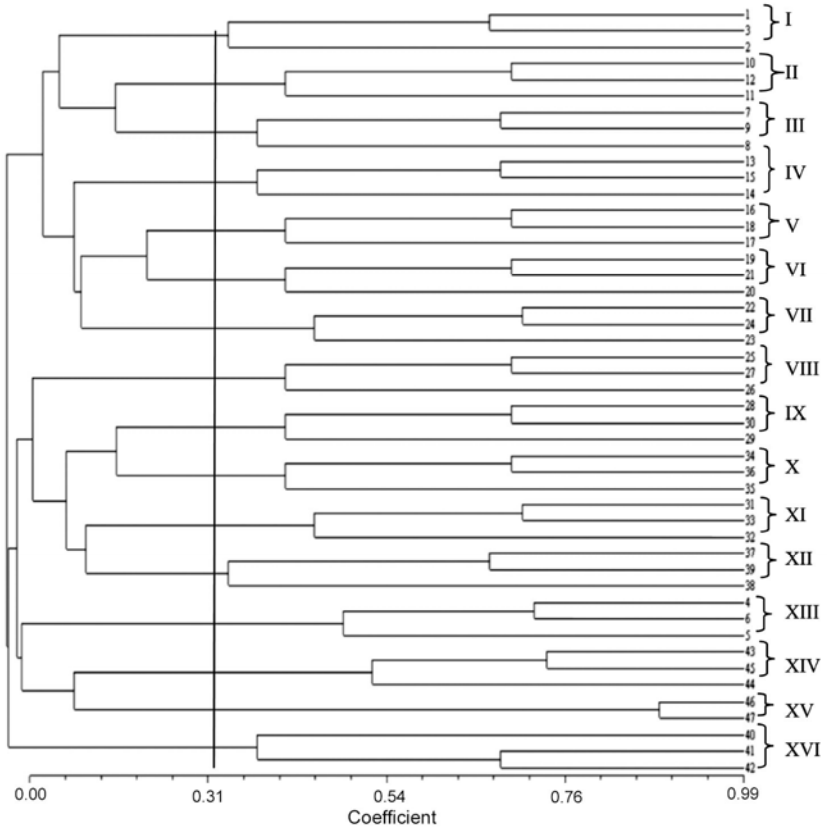

Fig. 3. A UPGMA cluster dendogram showing the genetic relationships among 47 approved hybrid rice genotypes.

Among the different markers, SSR markers are useful for a variety of applications in seed purity studies due to their reproducibility, multiallelic nature, co-dominant inheritance, relative abundance and good genome coverage (Powell *et al*. 1996). 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) g) is being studied worldwide (Lee *et al.* 1996). 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. In the present investigation, 17 SSR primer pairs amplified a total of 272 alleles ranging from 10 to 23 alleles per primer. The average number of alleles per primer was 16. The Polymorphic Information Content (PIC) for these primers ranged from 0.86 to 0.95. The primers showed an average PIC value of 0.90 which confirms the fact that, the SSR primers used in this study were highly informative. In case of hybrids, F_1 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

Table 3. SSR marker alleles identified as molecular tags for rice hybrids using single marker.

SSR marker	Hybrid	Size of allele (bp)	
		CMS line	Restorer line
RM72	HB09F_1	174	184
RM584	Shakti F_1	132	120
RM248	BRRI hybrid dhan1	104	95
RM211	BRRI hybrid dhan2	168	149
RM219	BRRI hybrid dhan3	209	189
RM72	Heera2 F_1	166	172
RM128	Heera5 F_1	156	145
RM219	ACI1 F_1	201	185
RM211	ACI93024 F_1	153	172
RM584	LP70 F_1	134	124
RM248	LP106 F_1	111	101
RM219	LP108 F_1	199	222
RM20	Gold F_1	186	163
	Tia F_1	186	162
RM18	SL08 F_1	133	159
RM248	BRRI dhan28 (Check)	99	
	BRRI dhan29 (Check)	107	

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. Morphological plant evaluation is deficient in assessing the genetic purity of seed sample due to environmental effects of morphological traits. 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, Yun *et al.* 2005, Sundaram *et al.* 2007). At present, registration of new varieties is done based on DUS testing relying on morphological characters and protein profiles are also used to supplement morphological traits. Therefore, DNA markers are being contemplated for establishing DUS test. In the present study, 17 SSR markers were employed for distinguishing the 15 rice hybrids with their parental A and R lines including 2 inbreds and a molecular key was constructed based on these markers for identifying the genotypes. The uniqueness of the genotypes was also established by comparing with the fingerprints of the hybrids. Therefore, it is concluded that genetic purity analysis through microsatellites or SSR marker will remain a useful tool for resolving the problem arises in seed certification programme as well as the determination of genetic purity of the rice hybrids very quickly.

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