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Identification of Rice Hybrids and Restorer Line Using Microsatellite Markers

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A B S T R A C T

Twenty four hybrids developed from three CMS lines viz. IR79156A, CRMS 31A and CRMS 32A and eight diverse rice genotypes were included in the present study. 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. Out of sixteen, ten SSR markers produced polymorphic bands in the rice genotypes.RM171 and RM 216 were successfully recognized as hybrid and was discriminated from its parental lines (with same alleles).PCR based markers RM171 linked to *Rf4* respectively, found to be more accurate compared to other linked markers in identifying restorers and differentiating maintainers from others. Molecular screening with RM171 for fertility restoration can be a useful tool for identifying restorers from breeding lines of unknown restoration status with 100% efficiency without making and evaluating large number of test crosses. These markers are useful in marker assisted identification of *Rf* genes in back cross breeding program to develop near isogenic lines with multiple *Rf* genes towards the development of superior restorer lines.

Introduction

Rice yields have been reported to be stagnant due to genetic ceiling and it is opposite to human population which increases steadily. Alternative technologies are needed to overcome barriers to increase rice productivity viz. new plant type, hybrid rice and molecular breeding. Among these hybrid rice technology is an important approach for improving rice production. Whereas the first progeny will perform better than their parent lines. Hybrid rice known having 15-25% higher yield advantage than inbreed rice. This technology is not contributing to food security but also beneficial to the environment.

The fingerprinting of rice hybrids and identification of their genetic relationships are very important for quality hybrid seed and plant yield improvement. Accordingly, clear-cut identification of elite crop varieties and hybrids is essential for protection and prevention of unauthorized commercial use (Nandakumr *et al.*, 2004). In rice, it was reported that 1% impurity in hybrid seeds caused the yield reduction of 100 kg per hectare, hence, determination of seed genetic purity in hybrid crops is crucial to confirm the hybridization and resulted increased grain yields through heterosis.

Conventional characterization of hybrids based on specific morphological and agronomic data is time-consuming, restricted to a few characteristics, influenced by environmental condition and are 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.

SSR has much more polymorphism than most of other DNA markers, and is co-dominant and large in quantity. Therefore, SSR has become an ideal molecular marker for identification of plant variety and for genetic purity testing of hybrids

In hybrid rice technology maintainer and restorer lines will be identified by crossing parental (testers) lines with a CMS line and evaluating the F_1 for pollen and spikelet fertility. This system of restorer identification is time consuming and labour intensive. CMS line can be restored by nuclear genes governing fertility restoration (*Rf genes*) (Nematzadeh and Kiani, 2010). Among five fertility restorer genes identified for WA (wild abortive) cytoplasm. Among these*Rf3* and *Rf4* genes reported to be of more value for identification of restorers (Revathi *et al.*, 2013). Several DNA markers closely linked to *Rf* genes have been reported (Ahmadakhah *et al.*, 2007, Bazrkar *et al.*, 2008, Alavi *et al.*, 2009, Neeraja *et al.*, 2009, Sheeba *et al.*, 2009 and Grishma Shah *et al.*, 2012) which are useful in marker assisted identification of restorers in rice germplasm and further use in hybrid breeding program. This information in normal breeding the reported markers need to be validated. In order to utilize present study was undertaken to DNA profiling of hybrids rice, their parental lines and to screen of restorer line markers linked with fertility restoration.

Materials and Methods

The present investigation was conducted at Research cum Instructional farm and molecular biology laboratory of department of Genetics and Plant breeding, College of Agriculture, IGKV Raipur during *Kharif*, 2014.

Plant Material and Genomic DNA isolation

The experimental materials for the present investigation comprises of three CMS lines and eight testers. Total 24 hybrids were developed in line X tester fashion. Out of 24 hybrids, eight hybrids were included in the study. Details of these lines are presented in the Table 1.

Total rice genomic DNA extracted from young succulent, disease and insect free seedlings by mini prep method. Genomic DNA was isolated from young leaves of 10 plants of each parent lines while the leaves of individual plant were used for hybrids. DNA was extracted to given by Doyle and Doyle, 1987procedure with minor modification.

The extracted DNA content was quantified and parental polymorphism studies done through 16 SSRs primers. PCR mix for one reaction (volume 20 µl) contained 2 µl DNA, Sterile and Nanopure water 13.5 µl, 10x Assay buffer, 1 µl dNTP, 0.5 ul of each forward and reverse primer, 0.5 ulTaq DNA polymerase.

PCR Amplification was performed with the following steps: predenaturing at 94℃ for 4 min, followed by 35 cycles of 94℃ for 1min, 55℃ for 1min, and 72℃ for 2 min, and last step is 5min at 72℃.

Amplified products were analyzed using 5% polyacrylamide gel. Electrophores is done for 1hr at 199 volts. The gel along with the DNA

sample then stained with Eithidium bromide (10 µg/10ml) for 40-45 mins. Gel was visualized on UV- transilluminator and image can be seen in computer.

SSR assay

PCR analysis was done using a set of 16 SSR (simple sequence repeat) markers (Table 2) to identify the parental polymorphism between three lines (IR 79156A, CRMS31A and CRMS 32A and eight testers *viz*., Jawaphool, Swarna sub 1, R-1656-2816-9-2-3223-1, NPT-1, NPT-2, NPT-4, NPT-17, NPT-38. The genotypic dataset was generated based on the PCR amplification

Profile by scoring presence and absence of specific allele with specific base pair (bp) size for all the samples. Validation of linked markers was carried out using for identified restorers.

Results and Discussion

Eight hybrids along with their respective parents were analyzed with ten most informative *SSR* markers and hybrids, those showing bands for both the parents (cytoplasmic male sterile lines and restorer lines) were identified as pure hybrid.

Genetic purity testing

Being co-dominant, polymorphic SSR markers were used to test the seed purity of rice hybrids. As expected, when polymorphic bands detected for two parent [CMS line (A) and restorer line(R)], two alleles were observed in F_1 hybrid individuals, while off type seeds had only one allele. In this study, Seed purity test was carried out with individual seedlings in each hybrid by respected polymorphic markers.

Table.1 Rice hybrids and their parental lines used in this study

S. No.	Hybrids / Parent lines	Type	Source
1	IR 79156A (WA)	CMS	IRRI, Manila, Philippines
2	CRMS 31A (Kalinga)	CMS	CRRI, Cuttack
3	CRMS 32A (Kalinga)	CMS	CRRI, Cuttack
4	Jawaphool	Tester	IGKV, Raipur, C.G.
5	Swarna-sub-1	Tester	CRRI, Cuttack
6	R-1656-2816-9-3223-1	Tester	IGKV, Raipur, C.G.
7	NPT-1	Tester	IGKV, Raipur C.G.
8	NPT-2	Tester	IGKV, Raipur C.G.
9	NPT-4	Tester	IGKV, Raipur C.G.
10	NPT-17	Tester	IGKV, Raipur C.G.
11	NPT-38	Tester	IGKV, Raipur C.G.
12	IR79156A / Swarna sub 1	Hybrid	IGKV, Raipur C.G.
13	IR79156A / NPT-38	Hybrid	IGKV, Raipur C.G.
14	CRMS31A / R-1656-2816-9-3223-1	Hybrid	IGKV, Raipur C.G.
15	CRMS31A / NPT-4	Hybrid	IGKV, Raipur C.G.
16	CRMS31A / NPT-17	Hybrid	IGKV, Raipur C.G.
17	CRMS32A / Jawaphool	Hybrid	IGKV, Raipur C.G.
18	CRMS32A / NPT-1	Hybrid	IGKV, Raipur C.G.
19	CRMS32A / NPT-2	Hybrid	IGKV, Raipur C.G.

Table.2 List of SSR markers used to detect polymorphism among the parents (A and R lines)

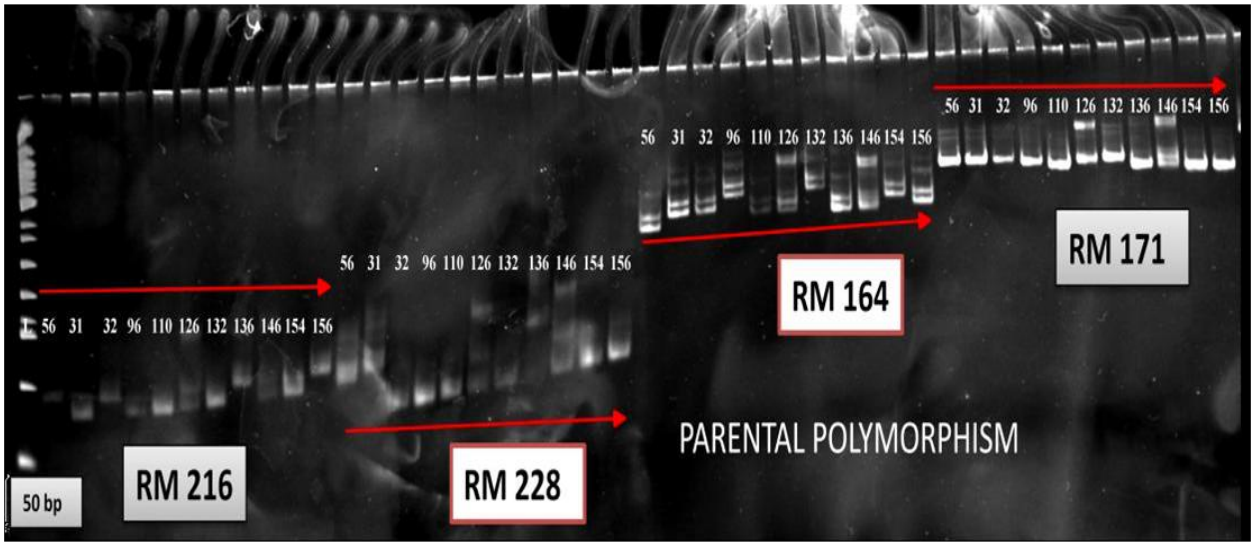
S. No.	Primer	Forward/ Reverse	Sequence 5'----------> 3'	Polymorphic/ Monomorphic	Position
1	RM-1	**Forward**	GCGAAAACACAATGCAAAAA	**Polymorphic**	1
		Reverse	GCGTTGGTTGGACCTGAC		
2	RM-154	**Forward**	ACCCTCTCCGCCTCGCCTCCTC	**Polymorphic**	2
		Reverse	CTCCTCCTCCTGCGACCGCTCC		
3	RM-263	**Forward**	CCCAGGCTAGCTCATGAACC	**Polymorphic**	2
		Reverse	GCTACGTTTGAGCTACCACG		
4	RM55	**Forward**	CCGTCGCCGTAGTAGAGAAG	Monomorphic	3
		Reverse	TCCCGGTTATTTTAAGGCG		
5	RM 514	**Forward**	AGATTGATCTCCCATTCCCC	Monomorphic	3
		Reverse	CACGAGCATATTACTAGTGG		
6	RM-168	**Forward**	TGCTGCTTGCCTGCTTCCTTT	**Polymorphic**	3
		Reverse	GAAACGAATCAATCCACGGC		
7	RM 307	**Forward**	GTACTACCGACCTACCGTTCAC	Monomorphic	4
		Reverse	CTGCTATGCATGAACTGCTC		
8	RM 124	**Forward**	ATCGTCTGCGTTGCGGCTGCTG	Monomorphic	4
		Reverse	CATGGATCACCGAGCTCCCCCC		
9	RM-164	Forward	TCTTGCCCGTCACTGCAGATATCC	**Polymorphic**	5
		Reverse	GCAGCCCTAATGCTACAATTCTTC		
10	RM 454	Forward	CTCAAGCTTAGCTGCTGCTG	Monomorphic	6
		Reverse	GTGATCAGTGCACCATAGCG		
11	RM 510	Forward	AACCGGATTAGTTTCTCGCC	Monomorphic	6
		Reverse	TGAGGACGACGAGCAGATTC		
12	RM-337	Forward	GTAGGAAAGGAAGGGCAGAG	**Polymorphic**	8
		Reverse	CGATAGATAGCTAGATGTGGCC		
13	RM-171	Forward	AACGCGAGGACACGTACTTAC	**Polymorphic**	10
		Reverse	ACGAGATACGTACGCCTTTG		
14	RM-216	Forward	GCATGGCCGATGGTAAAG	**Polymorphic**	10
		Reverse	TGTATAAAACCACACGGCCA		
15	RM-228	Forward	CTGGCCATTAGTCCTTGG	**Polymorphic**	10
		Reverse	GCTTGCGGCTCTGCTTAC		
16	RM-6100	Forward	TCCTCTACCAGTACCGCACC	**Polymorphic**	10
		Reverse	GCTGGATCACAGATCATTGC		

Table.3 Pollen fertility and spikelet fertility percentage of different CMS based hybrids in rice

S.No.	Genotypes	IR-79156A		CRMS 31A		CRMS 32A	
		SF %	**PF %**	**SF %**	**PF %**	**SF %**	**PF %**
1	**Jawaphool**	67.81	69.50	68.19	70.74	42.33	44.71
2	Swarna sub-1	76.54	*78.54*	63.66	64.92	72.51	74.7.56
3	R-1656-2816-9-3223-1	61.61	62.67	78.28	62.67	67.88	70.60.22
4	NPT-1	60.0 1	59.40	62.13	63.71	66.40	68.72
5	NPT-2	75.87	78.77	77.47	79.71	49.28	54 34.01
6	NPT-4	77.47	80.07	**79.32**	**81.32**	**77.15**	**82.51**
7	NPT-17	50.12	53.66	77.11	79.73	52.67	50.59
8	NPT-38	73.24	75.77	76.60	78.46	68.85	70.53

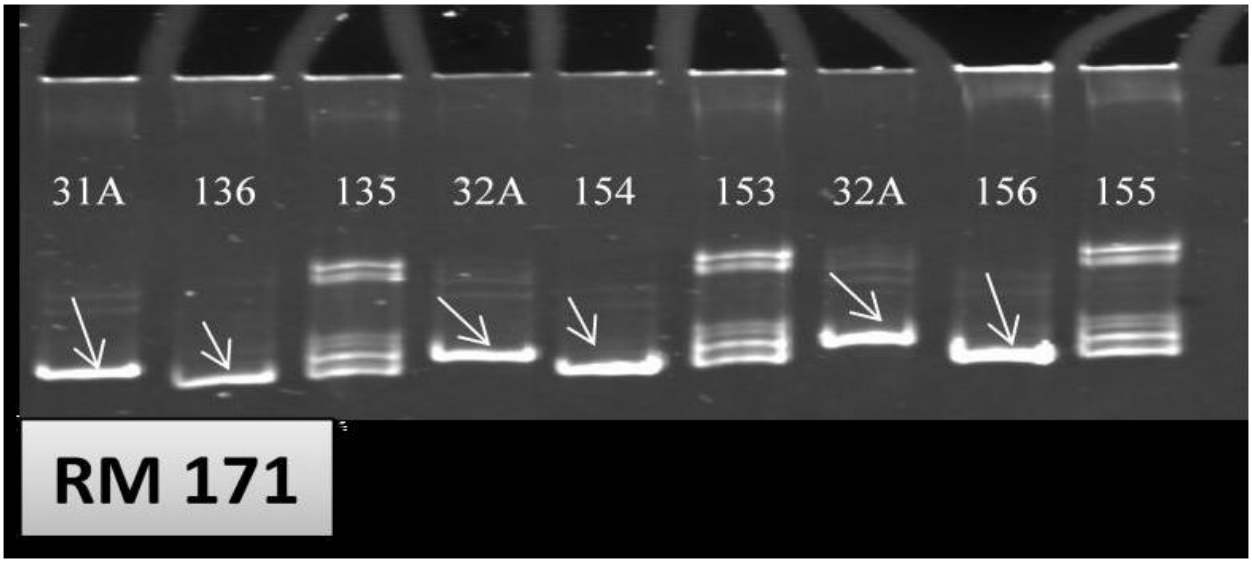
Note: (Pollen fertility % >80 and spikelet fertility % >75) Potential Restorer

Table.4 Gel image label description of Fig.1



56	IR79156A
31	CRMS31A
32	CRMS32A
96	Swrna sub 1
110	NPT 38
126	R 1656-2816-9-3223-1
132	NPT-4
136	NPT-17
146	Jawaphool
154	NPT 1
156	NPT 2

Table.5 Gel image label description of Fig.2



31A	CRMS 31A
136	NPT-17
135	CRMS 31A × NPT-17
32A	CRMS 32A
154	NPT-1
153	CRMS 32A × NPT-1
32A	CRMS 32A
156	NPT-2
155	CRMS 32A × NPT-2

Out of ten polymorphic markers, only two primers viz. RM 216 and RM171 have shown bands in both the parents (A lines and R lines), which indicating their absolute purity. Two primers were amplified as both alleles of their respective parents in RM 216 for a hybrid (IR 79156 A x Swarna Sub-1) hybrid and RM 171 for three hybrids viz. CRMS 31A/NPT-17, CRMS 32A/NPT 1 and CRMS 32A/NPT2, (Figs. 1 and 2). Identification and use of such hybrid specific markers can effectively reduce the cost and simplify the procedures of hybrid identification. Maintenance of hybrid seeds conformity in high level is essential for exploitation of hybrid vigor. Therefore, testing the hybrid seed purity is necessarily required before its release into the market. RM216 and RM171 markers for genetic purity test for three hybrids were recomended. Similar findings also reported by Yashitola *et al.,* (2002), Rajendrakumar *et al.,* (2007), Nandkumar *et al.,* (2004) and Sundaram *et al.,* (2008).

One DNA markers (RM 171) reported to be linked with restorer genes *Rf4* of WA cytoplasm among eight tester lines. In present study RM171 showed efficiency in restorer identification whereas non-restorers also identified with higher selection accuracy in comparison with pollen and spikelet fertility (Table 3). These results are in close confirmation earlier reports Hashemi *et al.,* (2013) found PCR based markers RM171 and exhibiting efficiency in restorer identification. In the present study heterozygous individual, RM171 and RM 216 were successfully recognized as hybrid and was discriminated from its parental lines (with same alleles) and molecular screening with RM171 for fertility restoration can be a useful tool for identifying restorers from breeding lines of unknown restoration status with 100% efficiency without attempting and evaluating large number of test crosses. Thus use of molecular markers linked to *Rf* genes would save time and cost besides adding accuracy in identification of restorers. This marker are useful in marker assisted identification of *Rf* genes in recurrent back cross breeding program to develop near isogenic lines with multiple *Rf* genes towards the development of superior restorer lines.

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