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Optimization of the choice of molecular markers for identification of commercially used rice varieties in India using rapid DNA extraction protocol

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Abstract: The present investigating aimed at the development of molecular marker for cultivar identification and genetic purity assessment. A total of four SSR markers and six SRAP primer were developed for the identification of sixteen different commercial varieties of rice. Traditional practice like grow-out-test based on morphological traits is time consuming and sometimes environmentally influenced. After development of molecular marker, it is using as an alternative to grow –out –test because of its rapid, accurate detection. We have assessed the potential of simple sequence repeat and sequence-related amplified polymorphism markers in distinguishing rice varieties and four simple sequence repeat markers namely CT-14, CT-25 CT XY-1 and ATC-3 and six sequence-related amplified polymorphism markers primers could be clearly distinguished sixteen commercially cultivar rice varieties. In addition to single markers, it's better to try with marker combinations, which were amenable for PCR and capable of distinguishing the varieties. Larger differences for each crop were found between cultivers from different seed companies than within the same company. These DNA markers can provide an easier and faster reliable genetic identification of rice cultivars.

Keywords: Molecular marker, *Oryza sativa,* Rice, Simple sequence repeat, Sequence-related amplified polymorphism

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the world's most important food crops, providing food for more than one third of the world's population. It is no longer a luxury food but has become the cereal that constitutes a major source of calories for the urban and the rural populations. Rice is grown in wide range of environments worldwide, even on a steep hill or mountain. Most of the world's rice is grown and consumed in Asia, which constitutes more than half of the global population. India is one of the Asian countries which had suitable agro-climatic conditions for rice growing. Approximately 11% of the world's arable land is planted annually to rice, and it ranks next to wheat. The world's rice production has doubled during last 25 years, largely due to the use of improved technology such as high yielding varieties and better crop management practices. Further scope of crop improvement depends on the conserved use of genetic variability and diversity in plant breeding programmes and use of new biotechnological tools (Chakravarthi and Naravaneni, 2006). Assessment of seed purity is one of the most important quality control components in hybrid seed production. Traditionally, it has been the practice to carry out a grow-out test (GOT), based on morphological traits, for assessment of purity of seeds. GOT takes one full growing season for completion, space demanding. It's often does not allow the unequivocal identification of genotypes. In the earlier reports, the use of Simple Sequence Repeat (SSR) and Sequence Tagged Site (STS) markers for rapid assessment of hybrid and parental line seed purity, as an alternative to GOT (Yashitola *et al.*, 2002; Yashitola *et al.*, 2004). Subsequently, Nandakumar *et al.* (2004) also showed the utility of SSR markers for fingerprinting rice hybrids. Dan *et al.* (2009) used Sequence-related Amplified Polymorphism (SRAP) to identify the hybrids and progeny of Zoysa crosses. Jing *et al.* (2012) provide a scientific basis for hybrid identification of switchgrass using SRAP and SSR marker. All these studies involved a limited of markers (<12) and the assessment of genetic purity was based on single marker and single seed or seedling based analysis. For accurate detection of impurities in seed lots, it is essential to identify a set of informative markers which can clearly distinguish the parental lines and amplify specific or unique allele combinations in the hybrids, not present in any other rice line. Moreover, the assay should preferably be based on analysis of bulked samples rather than single seed assays so as to bring down the cost of the assay (Sundaram *et al.*, 2008.). The aim of the present

study was to develop gene markers for cultivar identification and DNA fingerprinting, we have characterized 16 commercially use rice varieties using 20 uniformly distributed hyper polymorphic rice SSR markers and 10 SRAP markers and utilized them in varietal seed purity assessments.

MATERIALS AND METHODS

Sixteen variety of rice cultivars were collected form National Seed Corporation, New Delhi (Table 1). Genomic DNA was extracted using simple and rapid method for PCR analysis by Edwards *et al.* (1991) with slight modification. Fifteen seeds from each cultivar were kept in towel paper for germination inside a temperature controlled incubator. The temperature was set on 30-32^0 C. After 7-10 days germinated seeds were takeout. The plant tissue (100-200 mg) were cut into small pieces and place into a Mortar and Pestle. Add three times extraction buffer (cTAB) and grind it till a semisolid pest was seen. Extraction buffer was prepared with 10% CTAB, 1M Tris buffer, 0.5M EDTA beta-mercaptoethanol and distilled water. Keep it at pre-wormed water bath for 10-20min at 65^0 C. After taking out from water bath leave it for some time to cool at room temperature. Add equal volume of chloroform into the tube. Mix in a roto mix for 20 min followed by centrifuge at 13000 rpm for 10 minutes. Take out the aqueous layer in fresh eppendorf tube and add equal volume of Isopropanol and leave it for 30 min. Centrifuge at 13000 rpm for 10 min to get the DNA pallet. Decant the supernatant without disturbing the pallet followed by washing with 70% ethyl alcohol. Finally air dried the pallet and dissolved the pallet with 10:1 TE buffer (100 µl). To get more concentration dissolved the pallet 50-80 µl TE. The quality and concentration of extracted DNA were estimated by using a UV-Vis spectrophotometer (Nano drop, Thermo Fisher Scientific) at 260/280nm.

SSR amplification: DNA samples (40 ng) were amplified in 10-_l reaction volumes containing 1X PCR buffer [10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.01% (v/v) gelatin] (Bangalore Genei, India), 0.2 mM of each dNTPs (Bangalore Genei, India), 10 pmol of each primer and 1 U of Taq polymerase (Bangalore Genei, India). PCR was carried out in a Thermal cycler (BIOER Xp Cycler, China). The basic PCR profile was 5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at respective annealing temperature, 1 min at 72°C and 5 min at 72°C for final extension. The amplicons were resolved on 3% agarose gels, stained with ethidium bromide and visualized under UV as per the procedure described in Sambrook and Russell (2001) in a gel documentation system (Minilumi Gel Documentation System, Sigma-SVI) and impurities were identified based on deviations in expected amplification pattern.The sizes of the amplified fragments ere estimated with the help of Alphaease software utility of the gel documentation system using 1kb DNA ladders (MBI Fermentas, Lithuania) as the size standards. If a certain allele with respect to a particular SSR marker was observed uniquely in just one of the rice genotypes under study and absent in all the other rice genotypes, it was considered to be specific for that genotype and such SSR markers were categorized as informative SSR markers.

SRAP amplification: SRAP analysis was conducted according to previously established protocols (Li and Quiros 2001). In this assay, 30 different primer combinations were employed using six forward primers and six reverse primers combined randomly (Table 3). Firstly, SRAP primer combinations were screened. After screening under the optimized condition, primer combinations, generated strong and clear amplified bands, were selected for further investigations. Polymerase chain reaction was performed in 20 µL reaction mixture containing 1× buffer, 2.0 mM MgCl2, 0.2 mM dNTPs, 1 U Taq DNA polymerase, 0.5 µM primers, 50 ng DNA templates. Amplifications were carried out in a DNA thermal cycler (BIOER Xp Cycler) with an initial step at 94°C for 5 min, and five cycles of 1 min at 94°C, 1 min at 35°C, and 1.5 min at 72°C. The following 35 cycles consisted of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR reactions were repeated twice for each primer pair to ensure reproducibility. Amplified products were separated on 1.5% agarose gels in 1× TAE buffer using DNA marker as molecular weight marker and visualized with ethidium bromide staining.

RESULTS AND DISCUSSION

Germplasm characterization, diversity studies and seed purity assays, DNA fingerprinting approaches based on polymerase chain reaction have become methods of choice. A variety of DNA markers are now available for fingerprinting cultivars and for marker assisted selection. Of these, SSRs are the preferred ones for rice due to their abundance, co-dominant nature and their distribution throughout the genome and user-friendly nature (Mc Couch *et al.*, 2002). For the clear and stable amplification pattern SRAP marker is also effective for the authenticity, identification and genetic analysis (Dan *et al.*, 2009). The present study, we have assessed the potential of SSR and SRAP markers in distinguishing rice varieties and utilized 'informative' SSR and SRAP markers for testing purity of seeds. A set of twenty SSRs primer used for the present study. Selection was made from the literature and synthesized them on the basis of their amplification and banding pattern. Out of twenty SSRs primers four markers gave amplification of specific and unique alleles (Fig. 1) among the varieties. Four markers namely CT-14, CT-25 CT XY-1 and ATC-3 could be clearly distinguished sixteen commercially cultivar rice varieties. Therefore, these markers could be considered highly informative markers. Amplification was carried out with all four

Table 1. Details of the sixteen cultivars of rice with their parentage, type of variety and maturity days, used in the present study.

S.N.	Cultivars	Parentage	Hybrid/ Improved Variety	Maturity (In Days)
1	LALAT	(Obs 677/IR2071//Bikram W1263)	Improved	135
2	KRH-2	Hybrid (IR 58025A/KMR-3R)	Hybrid	125-130
3	COTTONDORA SANNALU (MTU-1010)	KRISHNAVANI/IR 64	Improved	120—120
4	RANJEET-2	(PANKAJ & MAHSURI)		155-160
5	MTU-7029	(VASISTA/MAHSURI)	Late	155-155
6	IR-64	(IR. 5857-33-2-1/IR.2061-465-1-5 5)	Improved	115-120
7	PANT-12	(GOVIND/UPRM 201-1-1)	Improved	115-122
8	DHANRAN	-------		
9	SAVITRI	(PANKAJ X JAGANNATH)		150-155
10	PB-1	----------	Improved	
11	JYOTHI	PTB 10 X IR-8	Improved	115
12	MTU-1031 (Tholakuri)	MTU 2077 / CR 316- 639	Late	150-155
13	KALA NAMAK	----------	Ancient Cultivar	145-150
14	VIJETHA (MTU-1001(1)	MTU 5249/MTU 7014	Improved	120-125
15	MTU-1001(2)	----------		
16	RANJEET-1	----------		

Table 2. SSRs Primers used in the present study.

S.N.	Primer ID	Primer Sequence	Annealing Temp (A^0)
1	CT 534	F ACAGTATTCCGTAGGCACGG R GCTCCATGAGGGTGGTAGAG	55
2	CT 522	F GCATGGCCGATGGTAAAG R TGTATAAAACCACACGGCCA	50
3	CT 404	F AACGCGAGGACACGTACTTAC R ACGAGATACGTACGCCTTTG	52
4	CT 360	F GGCTTCATCTTTGGCGAC R CCGGATTCACGAGATAAACTC	50
5	CT 199	F TCTCTCCTCTTGTTTGG CTC R ACACACCAACACGACCACAC	48
6	CT 125	F CCCATGCGTTTAACTAT TCT R CGTTCCATCGATCCGTATGG	48
7	CT 519	F CAAAAACAGAGCAGATGAC R CTCAAGATGGACGCCAAGA	50
8	CT 106	F ATCTTGTCCCTGCAGGTCAT R GAAACAGAGGCACATTTCATTG	55
9	CT 195	F GATCCAGCGTGAGGAACACGT R AGTCCGACCACAAGGTGCGTTGTC	55
10	CT 25	F GTACGACTACGAGTGTCACCAA R GTCTTCGCGATCACRCGC	55
11	CT XY1	F GTTGCGTCCTACTGCTACTTC R GATCCGTGTCGATGATTAGC	52
12	CT 368	F ATCAGCAGCCATGGCAGCGACC R AGGGGATCATGTGCCGAAGGCC	48
13	CT ATC3	F ACCCAACTACGATCAGCTCG R CTCCAGGAACACGCTCTTTC	50
14	CT 353	F CAACGTGATCGAGGATAGATC R GGATTTGCTTACCACAGCTC	48
15	GA 562	F TAGTGCCGATCGATGTAACG R CATATGGTTTTGACAAAGCG	50
16	GA 397	F TGTGAGCCTGAGCAATAACG R GAAGCGTGTGATATCGCATG	50
17	CT 115	F CGAGAGAGCCCATAACTACG R ACAAGACGACGAGGAGGGAC	55
18	GA 21	F TAGGTTGGCAGACCTTTTCG R GTCAAGATCATTCTCGTAGCG	48
19	GA 3	F TTGTCAAGAGGAGGCATCG R CAGAATGGGAAATGGGTCC	52
20	CT 131	F GGCTTCATCTTTGGCGAC R CCGGATTCACGAGATAAACTC	50

Table 3. SRAP primer combination and their sequence.

S.N.	Primer Id	Primer sequence (5'to 3')	Bases
1	Me 2 + Em 2	TGAGTCCAAACCGGAGC + GACTGCGTACGAATTTGC	17 + 18
2	Me2 + Em 6	TGAGTCCAAACCGGAGC + GACTGCGTACGAATTGCA	17 + 18
3	Me 2 + Em 7	TGAGTCCAAACCGGAGC + GACTGCGTACGAATTCAA	17 + 18
4	Me 2 + Em 11	TGAGTCCAAACCGGAGC + GACTGCGTACGAATTCTA	17 + 18
5	Me 1 + Em 1	TGAGTCCAAACCGGATA + GACTGCGTACGAATTAAT	17 + 18
6	Me 1 + Em 3	TGAGTCCAAACCGGATA + GACTGCGTACGAATTAAT	17 + 18
7	Me 1 + Em 7	TGAGTCCAAACCGGATA + GACTGCGTACGAATTAAT	17 + 18
8	Me 1 + Em 9	TGAGTCCAAACCGGATA + GACTGCGTACGAATTAAT	17 + 18
9	Me 2 + Em 5	TGAGTCCAAACCGGAGC + GACTGCGTACGAATTAAT	17 + 18
10	Me 2 + Em 8	TGAGTCCAAACCGGAGC + GACTGCGTACGAATTAAT	17 + 18

Table 4. List of the SSRs markers with their unique amplification product. Product size measured in base pair (bp).

S. N.	Primer → / Variety ↓	CT-14	CT-25	CT XY-1	AT C -3
1	LALAT	186	172	260	225
2	KRH-2	186	136	270	240
3	MTU-1010	193	---	258	
4	RANJEET-2	179	----	255	235
5	MTU-7029	179	143	250	230
6	IR-64	186	158	225	220
7	PANT-12	179	143	250	215
8	DHANRAN	165	186	250	235
9	SAVITRI	179	186	235	225
10	PB-1	179	158	225	240
11	JYOTHI	150	179	250	200
12	MTU-1031	165	150	240	215
13	KALA NAMAK	158	155	275	235
14	MTU-1001 (1)	158	165	220	200
15	MTU-1001 (2)	----	----	---	235
16	RANJEET-1	150	193	235	230

Table 5. SRAP markers with their unique amplification product. Product size measured in base pair (bp).

S.N.	Primer Varety	Me2+Em5	Me2+Em8	Me1+Em1	Me1+Em3	Me1 + Em7	Me1+Em9
1	LALAT		1000				
2	KRH-2	100					
3	MTO-1010						350
4	RANJEET-2	800	400				
5	MTU-7029	1100					
6	IR-64					600	
7	PANT-12		550				
8	DHANRAN				250		
9	SAVITRI						
10	PB-1		450				
11	JYOTHI			980			
12	MTU-1031			300,400			
13	KALA NAMAK					930	1000
14	MTU-1001(1)				570		
15	MTU-1001(2)			550			
16	RANJEET-1			600			

polymorphic primers. Scored the bands with respect to molecular marker size is given in the table 4. Similar studies were reported in different rice varieties and hybrid by early workers (Nandakumar *et al.,* 2004; Tamilkumar *et al.,* 2009). Rapid molecular testing techniques for the verification of cultivar identity assume importance in the face of demands for exacting quality in the international trade, the need for assuring homogeneity in planting seed material and the protection of intellectual property rights (Singh *et al.,* 2007). Identification of genotypes based on the presence of unique alleles is of considerable importance as it can help the authorities in detecting any pilferage or mixing in seed lot (Aneja *et al.,* 2012). SRAP markers appear to be much more effective, quicker and less expensive to develop than SSR markers (Ahmad *et al.,*

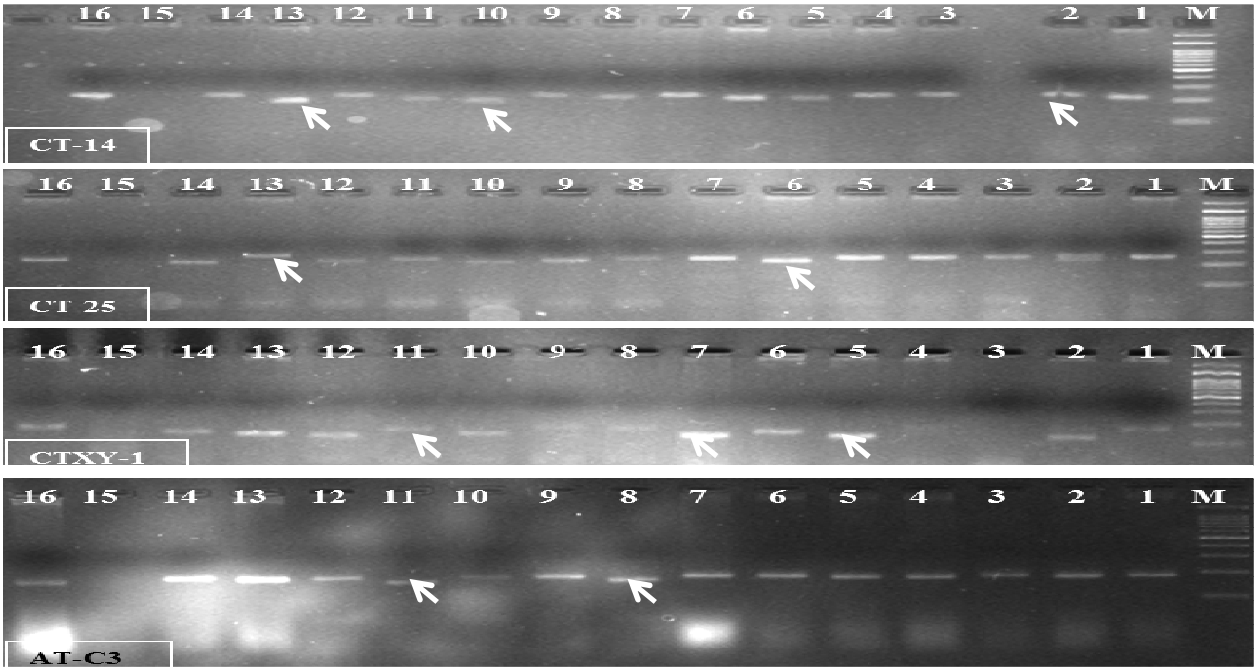

Fig. 1. *Agarose gel electrophoresis of a SSR-PCR reaction for primer CT-14, CT-25, CT XY-1 and AT-C3 with Rice DNA samples. Bands were fractionated by electrophoresis on a 3% agarose gel (1hr, 5V/cm, 1.0X Tris-Acetic acid EDTA buffer) and visualized by Ethidium bromide staining. M: 1Kb ladder (MBI Fermentas, Lithuania). Lanes: 1.Lalat, 2.KRH-2, 3.MTU-1010, 4.Ranjeet-2, 5.MTU-7029, 6.IR-64, 7.Pant-12, 8.Dhanran, 9. Savitri, 10. PB-1, 11. Jyothi, 12. MTU-1031,13. Kala Namak, 14. MTU-1001(1), Mtu-1001(2) and Rajneet-1. Arrows indicate SSR* markers unique to particular cultivars.

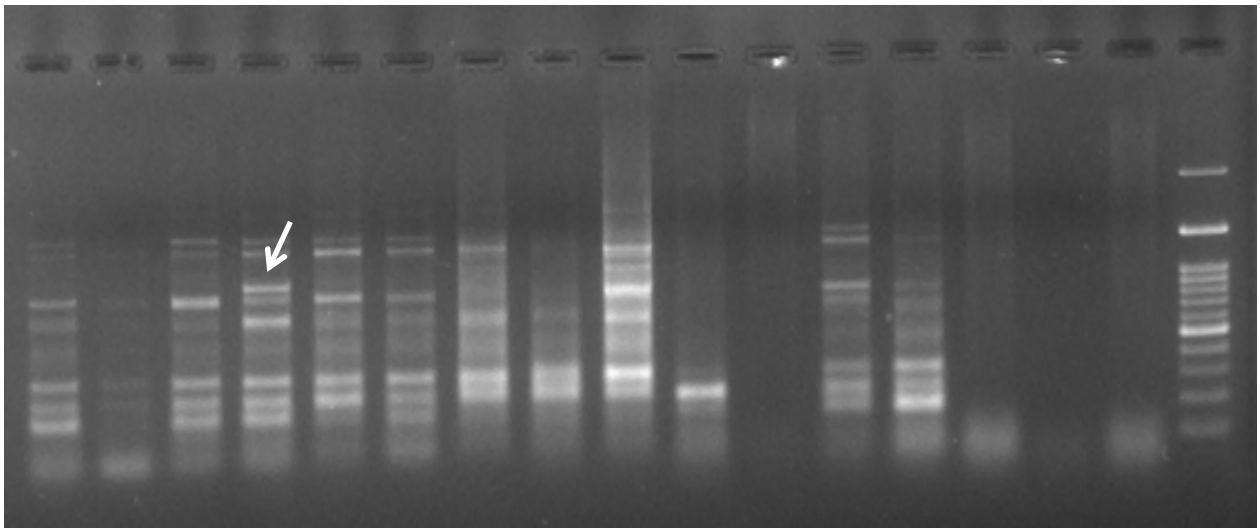

Fig. 2. *Sixteen rice cultivars showing the banding pattern with Me2 + Em5 and 8, SRAP marker. Bands were fractionated by electrophoresis on a 3% agarose gel (1hr, 5V/cm, 1.0X Tris-Acetic acid EDTA buffer) and visualized by Ethidium bromide staining. M: 1Kb ladder (MBI Fermentas, Lithuania). Arrows indicate SRAP markers unique to particular cultivars.*

2004). A set of twenty-two SRAP (Sequence Related Amplified Polymorphism) primer pair were selected for screening. Selection was made from the literature and synthesized them on the basis of their amplification and banding pattern. Out of twenty two SRAP primers six primers gave polymorphic result (Fig. 2). Scored unique bands with respect to their molecular size are given table 5. In addition to single markers, it's better to try with marker combinations (SSR + SRAP), which were amenable for PCR and capable of distinguishing the varieties. From the table 4 and table 5 such marker combinations can be used. Previous results indicated that SSR and SRAP markers can be used to distinguish the genetically very close cultivers as a complement to traditional pomological studies (Ahmad *et al.*, 2004). According to Sundaram *et al.* (2008) two informative microsatellite markers, RM164 and RM206 were used for assessment of purity of a

sample of KRH2 consisting of 400 seeds planted in a grow-out plot during wet season of 2005. Based on single marker and multiplex PCR analysis, RM164 identified eight contaminants and RM206 identified seven as contaminants. Though both the markers individually detected six contaminants, they differed in respect of the seedling numbers detected as contaminant, showing that screening using a single SSR marker may not be always accurate. Previous study revealed that a single polymorphic microsatellite marker might be sufficient for routine analysis of purity of commercial hybrid seed samples (Yashitola *et al.,* 2002). However, the additional information generated through the present study suggests that, in certain cases, the use of single microsatellite marker for purity testing may not be sufficient for accurate detection of impurities. Accurate marker combination and multiplex PCR which is a cost saving strategy, can be carried out simultaneously. Using the marker combination for varietal identification enhanced accuracy. Hence, we propose that analysis using single markers (as proposed by Yashitola *et al.,* 2002 and Nandkumar *et al.,* 2004) may not help in accurate estimation of seed impurities in certain cases, and wherever possible, it is better to deploy more than one marker or through multiplex PCR. A set of morphological descriptors are currently used for varietal identification, description and seed purity assessment. Though widely adopted and practiced, purity assessments based on morphology is often affected by environment, beside the on time and resources. Furthermore, many of the modern high yielding varieties and hybrids are phenotypically less distinct making morphological evaluation more difficult. Molecular markers have been used for genetic characterization of cultivars in rice, wheat, maize, sunflower and tomato (Sundaram *et al.,* 2008; Karkousis *et al.,* 2003; Wang *et al.,* 2002; Zhang *et al.,* 2005; Smith and Register, 1998). The Biochemical and Molecular Techniques Group of the International Union for the Protection of New Varieties of Plants (UPOV) is evaluating different DNA marker parameters prior to its routine use in establishing distinctness, uniformity and stability (DUS) of plant varieties (Bredemeijer *et al.,* 2002; UPOV-BMT, 2002). The present study we have established that selection of molecular marker are informative enough to distinguish all the rice cultivars. We have also identified 'cultivar specific microsatellite profile' for sixteen varieties which could be used for their seed purity estimation at different stages of seed multiplication (Tables 4 and 5).

Conclusions

With increasing number of public as well as private bred rice hybrids under commercial cultivation, quality control in terms of monitoring seed genetic purity at both parental and hybrid seed production stages is vital for the success of hybrid rice technology. Considering the innate disadvantages of GOT for seed purity analysis, marker based seed purity assay which could be an alternative, is receiving the attention. Replacement of GOT with a marker-based assay demands characterization of the parental lines with a large set of polymorphic markers to identify 'informative' markers. The present study is unique due to the fact that a comprehensive set of four Simple sequence markers and six Sequence Related Amplified Polymorphism markers can be used for distinguishing the commercially using cultivars to develop a marker database consisting of 'informative' SSR and SRAP markers. The utility of these markers in detection of impurities is also clearly demonstrated through cost saving strategies. The marker information developed through this study will be of immense help for rice seed industry to select appropriate marker combinations and assess purity at each stage of seed multiplication.

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