Research Article

Use of DNA polymorphism for characterization and assessment of genetic purity testing in Rice hybrids (*Oryza sativa* L.)

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Abstract
The investigation for genetic purity testing of hybrids in rice (*Oryza sativa* L.) using microsatellite markers was conducted on two rice hybrids (DRRH-2 and DRRH-3) and their corresponding parents (IR68897A, DR-714-1-2R, APMS-6A and RPHR-1005) at Seed Research and Technology Centre, Rajendranagar, Hyderabad during wet and dry seasons of 2011. The objective of the study is to identify the signature markers to differentiate parents and hybrids and also to replace grow out test, which has been used conventionally for seed purity testing through DNA based assays. 50 SSR markers were used to screen the cytoplasmic male sterile (CMS) lines, restorers and hybrids. A simple procedure has been standardized utilizing a two dimensional bulked DNA sample strategy involving a 20x20 grow out matrix which was designed for identification of contaminants in the seed lot of CMS lines, restorers and hybrids in 10 days old seedlings.

Key words: Hybrid rice, Genetic purity testing, Grow out test, SSR markers

Introduction
Rice is the major source of calorific needs of Asian population and it has been estimated that rice population in India as well as several other Asian countries must double by the year 2005 to meet the requirements of increasing population (Paroda, 1998). Hybrid rice cultivation offers an opportunity to increase rice yields and there by a steady supply of rice would be possible (Virmani and Kumar, 2004). Rice hybrids have been recorded a yield advantage of 15-20 per cent over semi- dwarf high yielding varieties (HYVs) in farmers fields (Mishra *et al.,* 2003). Maintenance of high level of genetic purity of hybrids is essential to exploit the moderate level of heterosis observed this crop. Growing popularity of commercial hybrids and the active involvement of public and private sector in large-scale hybrids seed production demands quality control in terms of monitoring seed genetic purity at both parental and hybrids seed production stage for success of hybrid technology. It is estimated that for every one per cent impurity in the hybrid seed, the yield reduction is 100 kg ha^{-1} (Mao *et al.,* 1996). Assessment of genetic purity plays a crucial role for successful commercialization of hybrids as seed quality directly influences the crop productivity. Further, the identification of hybrids, parental lines and determination of their genetic relatedness are very important for variety registration system, DUS testing and Protection of Plant variety and Breeder's rights (Ichii *et al*., 2003 and Kumar 1999). Unambiguous identification of elite crop varieties / hybrids and corresponding parental lines is essential for their protection and prevention of unauthorized commercial use (Nanda Kumar *et al.,* 2004).

Conventionally, the genetic purity of hybrid seed lots is assayed by a Grow Out Test (GOT), (which is purely based on phenotypic characters/ morphological markers) on a representative sample of seed that is to be marketed. GOT is time consuming and almost requires one full growing season for assessment of purity and involves lot of cost in terms of locked up capital and attendant problems of storage. Further, GOT is based on plant phenotypic characters and can be influenced by environmental conditions (Cooke, 1995).

The DNA based molecular markers are reliable and less time consuming, not stage or tissue specific and not affected by the environment. From the late 1980's different electrophoresis (Zillman and Bushuk, 1979; Tkachuk and Mellish, 1980) and reversed phase high performance liquid chromatography (RP- HPLC) (Scanlon *et al*. 1989) of seed storage proteins are well established and often considered effective method of cultivar identification. Currently, the simple sequence repeat (SSR) markers are most widely used (Sharopova *et al*., 2002; Maccaferri *et al*. 2007 and YU *et al*., 2011) for rapid assessment of hybrid and parental line seed purity. (Yashitola *et al*., 2002 and Sundaram *et al*., 2008). The utility of molecular markers in genetic purity testing of sunflower hybrids using EST-SSR markers were reported by Naresh *et al*. (2009) In rice, SSR markers are abundant and well distributed throughout the genome (Akagi *et al.,* 1996; Mc Couch *et al*., 1996). The current level of average genome wide coverage provided by microsatellite markers in rice, one marker for every centimorgans (Temnykh *et al.,* 2000) is sufficient to be useful for assessment of hybrid seed purity and for genotype identification. Therefore, the present investigation was to distinguish the parents and their hybrid combinations of DRRH-2 and DRRH-3 using a set

of 50 uniformly distributed hyper polymorphic rice microsatellite markers.

Material and methods
Two cytoplasmic male sterile (CMS) lines IR68897A and APMS6A, two restorer lines DR-714-1-R and RPHR-1005 and two hybrids namely, DRRH-2 and DRRH-3 released from Directorate of Rice Research were utilized in the current investigation.

DNA isolation**:** The genomic DNA of parents as well as their hybrids were extracted from the leaf samples after 10 days of sowing by Cetyl-Tri-methyl Ammonium Bromide (CTAB) method (Murray and Thompson, 1980) and the quantification was done by Nanodrop spectrophotometer (Thermo). The DNA was diluted in 0.1 T.E buffer to a concentration of 50 ng μl^{-1} for PCR analysis.

A total of 50 rice microsatellite loci were used for PCR amplification. The SSR markers were selected from the rice SSR linkage map (Temynkh *et al.,* 2000 and 2001). While selecting the primers sufficient care was taken to have the primers uniformly distributed across the 12 chromosomes of the rice genome. The primer sequence information was obtained from the published sequence data (www.Gramne.org). Further, primers having high polymorphic information content (PIC) were utilized for the analysis. Three μl of template DNA was pipetted into each of the PCR tubes after proper labelling and the PCR plate was kept at 4°C. Then the master mix was prepared by taking 0.5 μl of each primer (both forward and reverse primers), 0.5 μl of 2.5 mM deoxy ribonucleotides (dNTPs), 1 μl of Genei 10X assay buffer (10 mM Tris-Cl (pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ 0.01% gelatin) and 0.2 μl of 3 U/μl Taq DNA polymerase (Bangalore Genei Private Limited, Bangalore). Usually there will be minor errors while pipetting. Hence to avoid these errors, a little bit extra master mix was prepared to ensure that adequate quantity is available for all the samples. Empirically, for every 10 reactions, one extra reaction was taken. The temperature profile used for PCR amplification comprised 94^0C for 5min, followed by 35 cycles of 94^0C for 1min, 58^0C for 1min, 72^0C for final extension. The annealing temperature was adjusted based on the specific requirement of each primer combination. The PCR products were electrophoresed in 3% agarose gel at 120V and observed under gel documentation system (Syngene make).The sizes of the amplified fragments were estimated by using 50 and 100bp DNA ladders (MBL Fermentas, Ludhiana) as the molecular size standards. The markers exhibiting clear amplification of specific allele combination in hybrids and parental lines were considered as

informative SSR markers. The impurities were detected based on deviations in the expected amplification pattern.

Genetic purity assessment in A, R and hybrids**:**
A two dimensional strategy suggested by Nas *et al.,* (2002) was adopted for this purpose. Four hundred seeds each of A, R and hybrids were collected from commercial seed lot and were planted in a 20 row and 20 column matrix (Fig 5) during wet season, 2011 in the experimental plots of Seed Research and Technology Centre, Hyderabad, India. 10 days after sowing 2 cm leaf bits were collected from each of the seedlings on the rows of the matrix, bulked and used for isolation of a total genomic DNA by following the protocol of Zheng et al. (1995). Similarly, DNA was also isolated from bulked leaf bits of each column matrix, thus, a total of 40 DNA bulks representing 20 rows and 20 columns were prepared and these were amplified through PCR using informative markers RM 336 and RM 307 for DRRH-2 and DRRH-3, respectively.

The rows and columns, which showed different amplification pattern were considered as impure rows or columns. Plants located on hills where the impure row(s) and column(s) intersected were considered as suspected admixture(s). The seedlings in the 20x 20 grow out matrix was grown till maturity and the genotype as deduced from marker profile was verified with phenotype as deduced from GOT. The experiment was repeated once again in the dry season of 2011 and confirmed the results.

Results and Discussion
Out of 50 markers used RM336 and RM 307 were found to be polymorphic to distinguish DRRH-2and DRRH-3, respectively. The analysis using 50 evenly distributed hyper polymorphic SSR markers allow to identify the loci RM 336 specific or unique markers to characterize DRRH-2 and their corresponding parental lines IR68897A and DR-714-1-2R based on their banding pattern resolved on agarose gel (3%). Similarly, the marker RM 307 could clearly distinguish the hybrid DRRH-3 and their parental lines APMS-6A and RPHR-1005. The complementary banding pattern of the parents paved path towards the identification of these hybrids (Fig 1 and 2). The locus RM 336 amplified allele size at 190 bp was specific to female parent (IR68897A) and 170 bp was specific to male parent (DR-714-1-2R). These two bands of allele size 190 and 170 were found in hybrid DRRH-2. The other SSR primer RM 307 could able to distinguish the hybrid DRRH-3 by amplifying allele size of 120 bp, a female specific (APMS-6A) and an amplicon size of 160 bp a male specific allele (RPHR-1005).   Therefore, these markers could be considered informative markers for these hybrids.

Testing the Genetic Purity of Hybrid Seed**:**
To meet the standard specification of purity, the parental lines used in the hybrid seed production should have a very high level (99%) of purity (Yashitola *et al.,* 2002). Among the parental lines the purity of female line (CMS) is very crucial, as CMS lines can only be perpetuated by open pollination with its cognate isonuclear maintainer line (Sundaram *et al.,* 2008). Pollen contamination in seed multiplication plots of CMS lines is not uncommon. The marker, though highly useful in detecting the contamination of maintainer line seed stocks, cannot distinguish the contaminants where in the CMS line is pollinated by pollen shedders other than the cognate maintainer line. To address this problem two dimensional grow out matrix (Nas et al. 2002) was utilized.

A sample of 400 seeds were collected randomly from hybrid as well as parental lines seed lots of DRRH-2 and DRRH-3 and were grown in 20 x 20 matrix (20 rows and 20 columns). The leaf sample of 20 plants in each row and column were bulked. The DNA isolation was done from the bulked leaf sample of rows as well as column from DRRH-2, DRRH-3 and their corresponding female and male parents. These bulked DNA samples of DRRH-2 along with its parental lines were subjected to PCR analysis using microsatellite markers RM 336. Similarly, RM307 was utilized for genetic purity testing of DRRH-3 and its male and female lines. In the DNA profiling of hybrid DRRH-2, the hill at intersection of 4th row and 9th column were considered as off type (Fig 3, 4 and 5). It was observed that in female line of DRRH-2 (IR68897-A) 11th row and in 6th column amplified differently with RM 336 loci indicating the presence of off type in the female population (Fig 6 and 7), whereas in a DNA profile of 20x20 matrix of male parent of DRRH-2 (DR-714-1-2R), the plant at the intersection of 19th row and 7th column was confirmed as impure as it amplified differently (Fig 8 and 9). Similarly in case of DRRH-3 genetic impurity were also identified through the microsatellite loci RM 307 (Fig 10 to13). Similar type of genetic purity testing in rice using single marker was done by Yashitola et al,. (2002) and Nandakumar (2004). Further, more than one marker was (Multiplexing) employed by Sundaram *et al.* (2008) and they opined that PCR based analysis is a cost saving assay with enhanced accuracy.

It was observed that the variant plants identified on the basis of morphological characters also showed variation on molecular basis. Similar observations were made in rice (Kalaichelvan, 2009). Further, Keshavalu (2006) stated that the percentage contaminants detected based on SSR marker analysis was higher than those detected by GOT assay. In the current investigation also, molecular markers detected some additional impurities, which were not detected through analysis of morphological characters (GOT study). This shows the discriminatory power and efficiency of SSR markers in genetic purity testing and these markers could even accurately detect the residual heterozygosity in the seed. Earlier studies have shown that SSR markers are useful in identification and the assessment of hybrid and respective parent's plant to plant variation within parental lines and testing the genetic purity of rice hybrids (Yashitola et al. 2002 and Nandakumar et al. 2004)
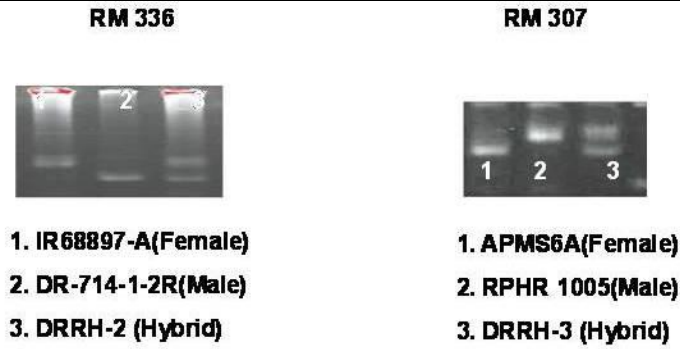
Conclusion**:** Based on the results of the present study, it could be concluded that SSR markers RM 336 and RM307 could efficiently used to generate molecular IDs for DRRH-2 and DRRH-3, respectively. Thus, these markers can be precisely used for assessment of seed purity to avoid GOT in order to save time and other resources. The SSR marker information developed through this study is helpful in the assessment of genetic contaminants in the seed supply chain and undertake necessary corrective action to supply the high quality seed to farmers. This assay can be used by the public and private seed companies for accurate and reliable detection of off- types in these hybrid seed lots for ensuring of good supply of quality seed to the farming community.
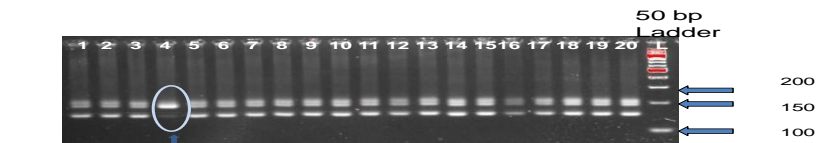
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RM 336

RM 307

1. IR68897-A(Female)

2. DR-714-1-2R(Male)

3. DRRH-2 (Hybrid)

1. APMS6A(Female)

2. RPHR 1005(Male)

3. DRRH-3 (Hybrid)

Fig 1, 2: Polymorphic SSR markers confirming hybridity of Rice hybrid DRRH-2, DRRH-3

Fig3: Testing the genetic purity of bulked seedling samples from 1-20 rows in DRRH-2, representing the off type in 4th row
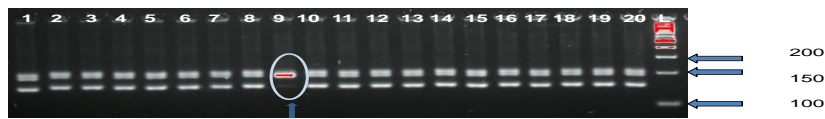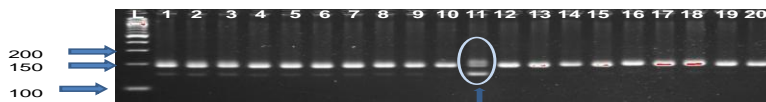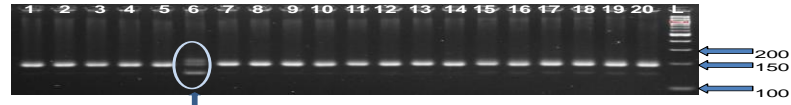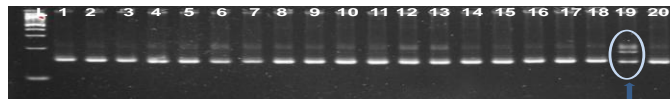
Fig 4: Testing the genetic purity of bulked seedling samples from 1-20 column in DRRH-2, representing the off type in 9th column
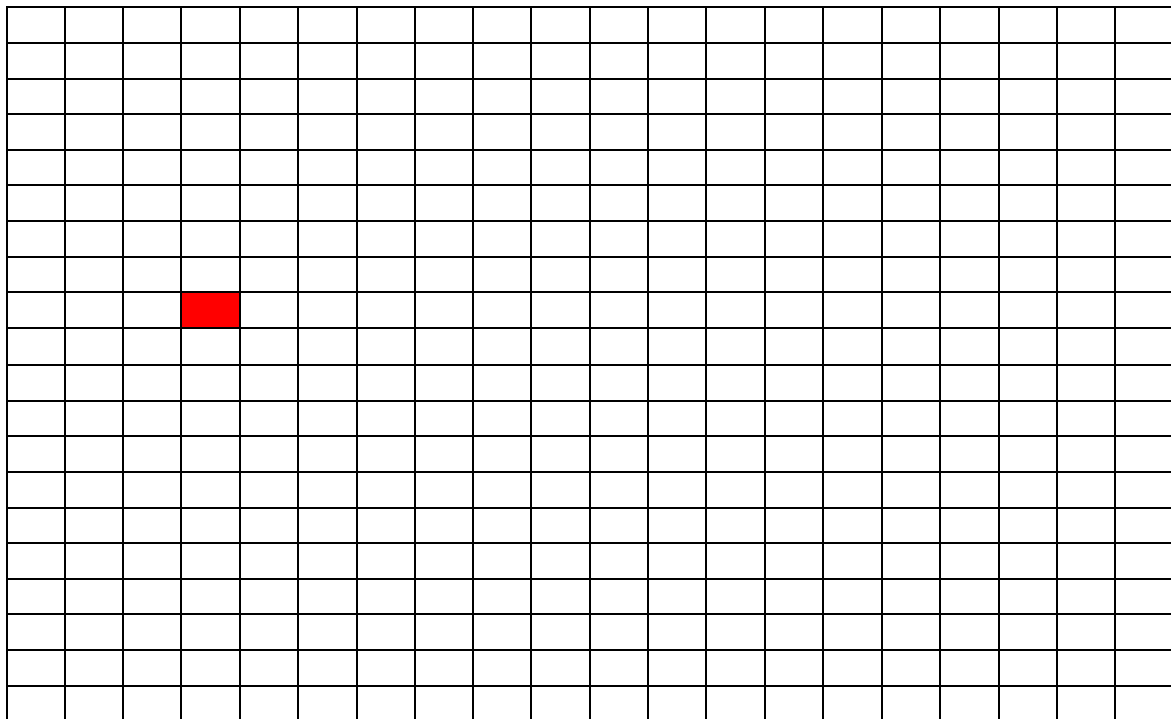
Fig 5: Schematic representation of 400 plants of hybrid rice DRRH-2 population representing the contaminant at intersection of 4th row and 9th column.
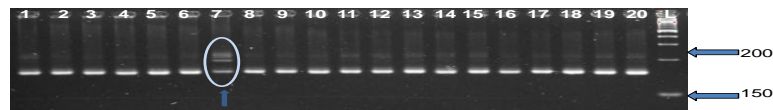
Fig 6 : Testing the genetic purity of bulked seedling samples from 1-20 rows in IR68897-A (Female line of DRRH-2), representing the off types

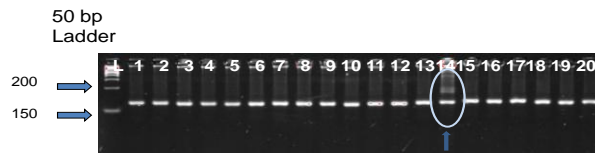

Fig 7: Testing the genetic purity of bulked seedling samples from 1-20 column in IR68897-A (Female line of DRRH-2), representing the off types
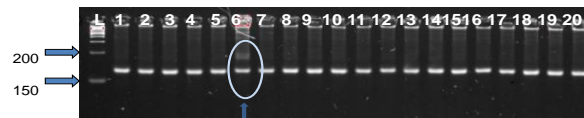

Fig 8 : Testing the genetic purity of bulke seedling samples from 1-20 rows in DR-714-2R (Male parent of DRRH-2) representing the off type at 19th row
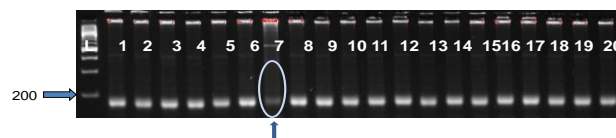

Fig 9 : Testing the genetic purity of bulked seedling samples   from 1-20 columns in DR-714-1-2R (Male parent of DRRH-2) representing the off type at 7th column

50 bp
Ladder

Fig 10 : Testing the genetic purity of bulked seedling samples from 1-20 rows in APMS6A, (Female parent of DRRH-3) representing the offtype at 14th row

Fig 11 : Testing the genetic purity of bulked seedling samples from 1-20 column in APMS6A, representing the off type at 6th column

50 bp
Ladder

Fig 12 : Testing the genetic purity of bulked seedling samples from 1-20 rows in RPHR 1005 (Male parent of DRRH-3) representing the off type at 7th row

Fig 13: Testing the genetic purity of bulked seedling samples from 1-20 column in RPHR 1005 , representing the off type at 8th column