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Short Communication

DNA fingerprinting and purity testing of the two pure line varieties of rice (*Oryza sativa*) using SSR markers

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About half of the world population depends on rice for their survival. It is cultivated in 114 countries, over an area of 161.4 million ha with the production of 466.7 million tonnes (On milled basis), but 90% of the world's rice is grown in Asia. According to the recent estimates, the world food production will have to increase by 70% by 2050 to meet the demand of ever growing population [1]. In order to narrow the gap between the production and demand, increase in productivity is the only option, since the other alternatives like expanding cultivable land and water are unavailable, and the other resources are either stagnant or declining [2].The cultivation of genetically improved varieties is the best way to get the higher yields. But, the yield potential of any variety can fully be exploited by ensuring the supply of genetically pure seed to farmers. Traditionally, genetic purity determination and varietal identification is being carried out by GOT which involves the evaluation of morphological or physiological traits expressed by seed, seedlings or mature plants. Being land and labour intensive, time consuming and influenced by environment, GOT need to be replaced by a simple, rapid, unbiased and cost-effective DNA based assay [3].

Therefore, molecular markers were employed as identification tools in the present study to overcome the disadvantages of conventional methods for varietal identification and genetic purity testing. These molecular markers are capable to change the entire methodology for seed purity analysis. The practical utility of molecular markers in present study has the potential to benefit all those involved in seed production chain. They can ensure the breeders and/or breeding institutions to get the benefits from the commercialization of varieties through a suitable system of PVP. For seed companies they can save millions of dollars involved in storage of seed and conduction of GOT. The farmers can be benefitted by timely availability of pure and fresh seed at low cost. In rice, microsatellites or SSRs' are abundant and well distributed throughout the genome [4, 5, 6, 7]. They are valuable as genetic markers because they are codominant, detect high levels of allelic diversity, and are assayed efficiently by the polymerase chain reaction [6]. The polymorphic survey of several microsatellite markers was conducted to identify SSR markers that can be used for identification and seed purity testing of the two popular varieties of rice.

The present study was conducted at molecular biology laboratory of the Department of Genetics and Plant Breeding, College of Agriculture, Indira Gandhi Krishi Vishwavidyalaya, Raipur (C.G.), (21^0 16′ N and 81^0 36′ E at an altitude of 289.60 m above mean sea level) during 2010. The genotypic data generated was used for purity assessment of seed lots of the two varieties under study. Two pure line varieties of rice *i.e.* Swarna and MTU-1010 were considered for molecular evaluation with respect to each other and with IR58025A (Table 1). These two pure lines are popular varieties and are under commercial cultivation at various locations in India. Due to the utilization of IR58025A and

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Table 1. Details of pure line varieties used in the study

Rice genotype with their parentage	Developed by	Salient Feature
IR58025A (IR-48483A/6 x Pusa 167-120-3-2)	International Rice Research Institute, Manila, Philippines	120 days duration, semi-dwarf, grains–LS, low amylose content (14.8 %), mild aroma, widely used CMS line, grain yield of respective maintainer line: 65 q/ha
Swarna or MTU 7029 (Vasisht x Mahsuri)	Andhra Pradesh Rice Research Institute, Maruteru (ANGRAU)	Duration 150 days, semi-dwarf, better grain quality, lodging resistance, brown glume, MS bran, high yielding, low input response, resistant to BLB, grain yield: 65 q/ha
MTU-1010 or Cottondora Sannalu (Krishnaveni x IR 64)	Andhra Pradesh Rice Research Institute, Maruteru (ANGRAU)	120 days duration, semi-dwarf (108 cm), grains–LS, white, resistant to blast and tolerent to BPH, grain yield: 74 q/ha

its easy availability in most of the hybrid breeding programmes, it was used as standard for comparison. IR58025A developed by International Rice Research Institute (IRRI), Manila, Philippines form the source of cytoplasmic male sterility and its maintenance in most of the commercial rice hybrids cultivated in India. The seed material used has been obtained from the Seed Production Farm of the Department of Genetics and Plant Breeding, College of Agriculture, Indira Gandhi Krishi Vishwavidyalaya, Raipur (C.G.).

Genomic DNA was isolated from young succulent diseases free leaves of 8-10 days old rice seedlings [8]. Quantification of DNA samples was done using Nanodrop Spectrophotometer, the DNA concentration was found to be in the range of 100-700 ηg/µl. The quantification was followed by dilution with nano-pure water to make the final working concentration of DNA @20 ηg/µl. The PCR amplification was done for each of the diluted DNA samples using 53 different types of primers. For this purpose, the 0.2 ml labeled PCR tubes containing 2 ml of separate working DNA solutions were added with the18 ml of the Cocktail (Reaction mixture), making the final volume as 20 ml. Standardization of the PCR protocol for SSR was done using various concentrations/quantities of primers, template DNA and *Taq* polymerase along with annealing temperature. PCR reaction mixtures were always prepared at sub-zero temperature by the sequential addition of various optimized reaction components (Table 2) for required number of reactions plus one reaction extra to compensate the pipeting loss.

The DNA amplification was carried out in PTC 100 (Programmable Thermo Cycler) of MJ Research Pvt Ltd, USA for 37 cycles. The temperature profile of Thermo Cycler was maintained as - 5 min of initial Denaturation at 94°C; 37 cycles each of

Table 2. PCR reaction components for one reaction

Reagent	Stock concen-tration	Volume (ml)
Sterile and nanopure H_2O	-	12.9
PCR buffer with 15 mM $MgCl_2$	10 X	2.0
dNTPs (Mix)	10 mM	1.6
Primer (forward)	10 µM	0.5
Primer (reverse)	10 µM	0.5
Taq polymerase	1 unit/µl	0.5
DNA template	20 ηg/ml	2.0
Total		20

Denaturation for 1 min at 94°C, Annealing for 1 min at 55°C and Extension for 2 min at 72°C; and final extension for 10 min at 72°C. The electrophoresis of the amplified DNA was carried out for 90 min at 130 volts on 6% polyacrylamide gel containing 10 ml of 10X TBE, 15 ml of 40% acrylamide solution (Containing acrylamide and bisacrylamide in the ratio of 19:1), 50 ml TEMED (N',N',N',N'-tetraethyl methylenediamine) and 600 ml of freshly prepared 10% APS in 100 ml of final solution. The polyacrylamide gel was stained with the help of ethidium bromide solution having 10 ml of 1% EtBr in 300 ml of distilled water. The gels were visualized on UV trans-illuminator and photographed by Sony Cyber Short Digital Camera having resolution of 7.2 Mega pixels. The DNA banding pattern of two pure line varieties of rice *i.e.* Swarna and MTU-1010 were compared with each other and with IR58025A. Fifty-three randomly selected microsatellite markers, evenly distributed throughout the rice genome were used in identification and fingerprinting for two pure line varieties of rice with respect to IR58025A and with each other. The variety Swarna exhibited polymorphism on 5 primers *i.e.* RM212, RM260, RM335, RM410 and RM444 with respect to line IR58025A, whereas only two markers *i.e.* RM212 and RM260 were identified as polymorphic in case of variety MTU-1010. The two pureline varieties exhibited the polymorphism on RM260, RM335, RM410 and RM444 with respect to each other. Swarna and MTU-1010 exhibited similar banding pattern on RM212, however both exhibited polymorphism with respect to IR58025A. Primers exhibiting polymorphism were screened twice to validate the results (Fig. 1).

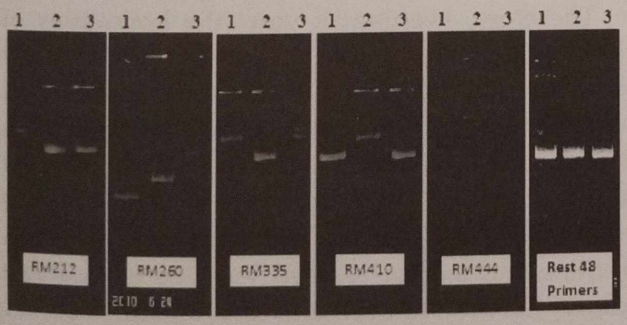

Fig. 1. Amplification pattern obtained using various SSR primers. The first five photographs show polymorphic banding pattern among different lines, whereas the last photograph shows monomorphic banding pattern (1 = IR58025A, 2 = Swarna and 3 = MTU-1010)

The frequency of heterozygosity in case of Swarna and MTU-1010 was 9.43% (5/53) and 3.77% (2/53), respectively with respect to IR58025A, with an average frequency of 6.60% (7/106). The two pureline varieties when compared with each other exhibited the polymorphism of 7.55% (4/53). The relatively low and high recovery of polymorphism under present study may be attributed to the narrow and broad genetic variation among the genotypes, respectively. The markers found to be polymorphic were used for assessing genetic heterogeneity of the commercial seed lot of variety Swarna. The base sequence of the primes used was obtained from the published sequence data (http://www.gramene.org/). The microsatellite markers exhibiting polymorphism in the present study were located on four chromosomes of rice genome (Table 3).

The SSR marker RM212 was used for accessing the genetic purity of commercial seed lot of variety Swarna (Fig. 2). In a random sample of 100 seeds, the markers identified only 4 off types indicating 96% genetic purity of seed lot. The results were confirmed with RM260 and RM410 using same stock DNA for amplification. For purity assessment, the DNA was isolated from the single seedlings of variety Swarna. Similar results were obtained when same seed lot of the variety was grown in the field and its morphological parameters were studied with the help of GOT.

The yield potential of any variety can fully be exploited by ensuring the availability of genetically pure and good quality seed to farmers. But, for seed certification, the only legally recognized traditional method of genetic purity assessment is based on field plot grow-out-tests which involve

Table 3. Location of microsatellite markers on different chromosomes of rice genome

Microsatellite locus	Location/Chromosome
RM212	1 (long arm)
RM260	11 (short arm)
RM335	4 (short arm)
RM410	9 (long arm)
RM444	9 (long arm)

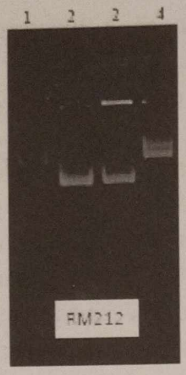

Fig. 2. Testing genetic purity of the seed lot of Swarna using SSR marker RM212. The first band represents IR58025A, second and third bands represent Swarna, whereas as the fourth band shows the presence of off-type (*i.e.* 1 = IR58025A, 2 = Swarna, and 4 = off- type)

only the morphological characteristics of a variety. The morphological traits are mainly multigenic or quantitative and their expression is altered by environmental factors. They require replications of observations, trained personnel, suitable land and time to record the data. Thus, we need to develop the methodology for testing the seed purity that is accurate, cheap and faster. The DNA-based markers have potential to serve the purpose as they can ideally be used for precisely assessing the genotype of a plant. The SSR markers have characters like reproducibility, stability over different environments, no plant stage specificity, multiallelic nature, co-dominant inheritance, relative abundance, good genome coverage which can serve them as convenient and reliable tools for varietal identification and purity testing according to the international norms. There are currently at least 2200 well distributed and mapped microsatellite markers in rice which constitute a large source of markers for detecting polymorphisms between any two genotypes of rice [9].

Using molecular markers for testing genetic purity substantially reduces the time, space, labour and the cost involved in testing. Therefore, the problems arising in seed certification programmes can be resolved by genetic purity analysis through SSR marker. Identification of rice varieties and testing their genetic purity plays a significant role in the issues related to plant variety registration and plant breeders' rights. The marker(s) used for testing the varietal purity should exhibit distinguish banding pattern. The polymorphism obtained using particular marker should be characteristic feature of that variety and should not match with any other varieties. These polymorphic markers can be identified in polymorphism surveys conducted by the random use of either microsatellite or STS markers on the varieties and their assumed standard lines. Establishment of the expected polymorphism can then be used for the varietal purity testing.

Use of two polymorphic markers is generally sufficient to ascertain the seed purity but more than two markers can be used to confirm the polymorphic results subjected to that additional cost. Multiplex PCR using two different primers simultaneously can be used to reduce the expenditure. A cost of approximately US$ 2 (Rs.120) per marker per seedling was estimated which includes the costs of reagents for DNA isolation, PCR amplification and polyacrylamide gel electrophoresis. This would increase to about US$ 2.5 (Rs. 150), if two markers were multiplexed. However, this cost excludes the labour and overhead charges involved. As far as the manpower is concerned, three technicians working in a modestly equipped laboratory can completely assay 200 seedlings within about 40 working hr. If suitable number of trained persons and machinery is involved, the DNA test can be completed within 15 to 20 days from the date of harvest, so that the seed produced in *rabi* season can be used for commercial cultivation in the ensuing *kharif* season. This will lead to the considerable savings involved in storage of seed and growing the crop for GOT.

Therefore, keeping in view the limitations of traditional techniques for assessing the varietal genetic purity, present study was conducted to take the advantage of molecular markers as identification tools, which are capable to change the entire scenario for seed purity analysis. The DNA-based markers are versatile tools in DNA profiling and marker assisted selection of desirable genotypes. They can serve as best means to ensure the breeders and/or breeding institutions could get the due benefits from commercialization of varieties through a suitable system of PVP. For seed companies they can save millions of dollars involved in seed storage and conduction of GOT.

The farmers can be benefitted by timely availability of fresh and pure seed at low cost. In short, the practical utility of molecular markers in present study will benefit all those involved in seed production chain.

REFERENCES

1. FAO (Food and Agriculture Organization of the United Nations) (2010). FAOSTAT

2. YASHITOLA J, THIRUMURUGAN T, SUNDARAM RM, NASEERULLAH MK, RAMESHA MS, SARMA NP AND SONTI RV (2002). Assessment of purity of rice hybrids using microsatellite and STS markers. *Crop Sci* **42:** 1369-73

3. NARESH V, YAMINI KN, RAJENDRAKUMAR P AND KUMAR VD (2009). EST-SSR marker-based assay for the genetic purity assessment of safflower hybrids. *Euphytica* **170:** 347-53

4. AKAGI H, YOKOZEKI Y, INAGAKI A AND FUJIMURA T (1996). Microsatellite DNA markers for rice chromosomes. *Theor Appl Genet* **93:** 1071-77

5. MCCOUCH SR, PANAUD O, CHEN X AND XU Y (1996). Development of microsatellite markers and characterization of simple sequence length polymorphims in rice (*Oryza sativa* L). Khush GS (ed) *Rice Genetics III: Proceedings of Third International Rice Genetics Symposium.* Los Banos Manila, the Philippines, International Rice Research Institute pp 549-59

6. MCCOUCH SR, CHEN X, PANAUD O, TEMNYKH S, XU Y, CHO YG, HUANG N, ISHII T AND BLAIR M (1997). Microsatellite marker development, mapping and application in rice genetics and breeding. *Pl Mol Biol* **35:** 89-99

7. WU KS AND TANKSLEY SD (1993). Abundance, polymorphism and genetic mapping of microsatellites in rice. *Mol and Gen Genet* **241:** 225-35

8. XU X, KAWASAKI S, FUJIMURA T AND WANG C (2005). A protocol for high throughput extraction of DNA from rice leaves. *Pl Mol Biol Reporter* **23:** 291-95

9. TAMILKUMAR P, JERLIN R, SENTHIL N, GANESAN KN, JEEVAN RJ AND RAVEENDRAN M (2009). Fingerprinting of rice hybrids and their parental lines using microsatellite markers and their utilization in genetic purity assessment of hybrid rice. *Res J Seed Sci* **2:** 40-47.