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Testing seed genetic purity in rice using SSR markers

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Abstract
The present study was undertaken with an aim of optimizing SSR marker technology for detecting admixtures and fixing threshold limits of admixture that can be detected using SSR makers. Seeds of two varieties were mixed together in four different ratios and genotyping was carried out using polymorphic SSR markers. The results indicated that the 24:1 ratio i.e. 5% admixtures or offtypes can be detected using this method. The results were further confirmed in other set of varieties and pooling ratios. Results confirmed that this method can be used for detecting offtype admixtures in seed lots using SSR markers. The efficiency of the method can further be increased by increasing the DNA concentration and PCR cycles in the PCR reactions. Thus this method would help in detecting the admixtures in the seed lot and would further help in replacing the present GOT thereby reducing the time, space, labour and especially the environmental influence.by that dilution of phenol, which disinfects the suspension in same time, determines its phenol coefficient.

Keywords: Testing seed, rice using, detecting admixtures

Introduction
The advent of high yielding varieties during Green Revolution has doubled rice production. This improved rice production not only made countries like India able to achieve self-sufficiency in rice, but also a net exporter of rice. However, rice demand is expected to increase dramatically in the near future with the growing population. The expected production can be achieved through the use of high yielding improved varieties. The success of improved variety in the farmer's field depends on the availability of seeds with high genetic purity (Agarwal, 1999) [1]. Because seed carry the genetic information determining yield and other agronomic traits, genetic purity of seed is important in harvesting good crop. The demand for high quality seeds is increasing day by day and it is a huge challenge for seed industry to maintain the varietal purity in larger area during seed production. In a country like India, where certified seed production is done by registered farmers and private sector (Mishra *et al*., 2003) [9], monitoring genetic purity at each stage of seed production becomes necessary. So, without a planned method for maintaining genetic purity, there is a grave danger of losing varietal identity.

Seed testing for quality assurance is one important step in the process of production of high quality seed. Traditionally the genetic purity of seeds has been assessed by Grow Out Test (GOT), based on morphological traits. Characterization based on morphological markers not only time taking and laborious but are also less reliable as many characters of interest have low heritability and genetically complex (McDonald, 1995) [8]. Besides that, the time required to grow and evaluate a large number of seed lots may be a major limitation to many seed testing laboratories, particularly if mature plants are needed for that. A few cultivars may be easy to distinguish one from each other by visual observation, but keeping track of many commercial inbreds, hybrid varieties and landraces makes the task dauntingly inefficient. In recent years, several new cultivars have been produced from hybridizations between members of a genetically very close elite group. This will further complicate the task of unambiguously identifying new varieties by the use of conventional characteristics alone. As the number of varieties increases, the ability to distinguish them on the basis of selected traits becomes more difficult. In contrast to morphological traits, molecular markers can reveal abundant difference among genotypes at the DNA level, providing a more direct, reliable and efficient tool for assessing the genetic purity of the variety.

Furthermore, DNA markers are 'neutral', and they have no effect on phenotype, no epistatic effect, and are not influenced by environmenta conditions and developmental stages. According to Dwiatmini *et al*. (2003) [4], molecular markers can provide illustration about the more accurate genetic relationship.

Ravi *et al*. (2003) [11] suggested that the SSR marker analysis based on the availability of more than 2500 SSR loci covering the entire rice genome (Temnykh *et al*., 2000; McCouch *et al*., 2002) [13, 7] can remain as the future viable strategy for the marker based varietal profiling and purity analysis in rice. The present study was undertaken with an aim of identifying threshold limit of seed admixtures that can be detected through molecular method using SSR markers.

Materials and Method

Seed material and DNA extraction

The pure seeds of four rice varieties were collected from the Paddy Breeding Station, Tamil Nadu Agricultural University, and Coimbatore. DNA was extracted from paddy seeds. Six different sets of DNA extraction were performed. Seeds of two rice varieties were purposefully mixed in 4 different ratios to estimate the level of contamination that can be detected using molecular markers i.e. 9:1, 24:1, 49:1 and 99:1 (i.e., 9 seeds of one variety+ one seed of other variety).

DNA isolation for seeds of paddy variety

Genomic DNA was isolated from seeds by mini-prep CTAB method as described by Doyle (1987) [3] with minor modifications. Seeds were homogenized along with 600 µl of pre-heated extraction buffer (2%CTAB, 100 mM Tris–HCL pH 8.0, 50 mM EDTA pH 8.0, 1.5 M NaCl) using tissue lyzer (Qiagen, USA). The tubes were placed in a 65°C water bath for 30 min with occasional mixing. The above sample was extracted with equal volume of chloroform: isoamyl alcohol (24:1) and supernatant was transferred in to a new tube. The sample was treated with RNase and extracted further with chloroform: isoamyl alcohol (24:1) and precipitated with 80% of ethanol. The pellet was air dried and dissolved in 100 µl of Milli Q water. DNA concentration and purity based on A260/A280 ratios was quantified using a Nano-Drop spectrophotometer (ND-1000 Spectrophotometer, Nano Drop Technologies, USA) and the DNA concentration was normalized to be 25 ng/µl for PCR reaction.

PCR analysis using DNA samples of pooled seed samples

DNA samples obtained from pooled seeds of two varieties in different ratios viz. 9:1, 24:1, 49:1 and 99:1 were diluted to 50 ng/µl and were subjected to PCR analysis using identified polymorphic SSR markers between two genotypes. A total of 57 microsatellite markers were used detect the admixture in the seeds mixed in different ratios. PCRs were performed in 15 µl reactions containing 50 ng of DNA template, 1X Taq buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM primer, 1 unit Taq DNA polymerase (Thermo Scientific, USA). Amplification was performed in programmed thermal cycler i.e., MyCycler (BioRAD, USA) with program of initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, primer annealing at 55°C for 1 min, extension at 72°C for 30 sec and final extension at 72°C for 10 min. Amplification products were electrophoresed in 3% agarose in TBE (90 m M Tris–borate, 2 mM EDTA, pH 8). The gels were stained with ethidium bromide and documented using gel documentation system (Bio RAD, USA).

Results and Discussion

Any crop yield is determined by the genetic purity of the seed used for planting. The genetic purity of any agricultural crop propagated by seed begins with the purity of the seed planted. The genetic purity of the seed planted must equal or exceed the final product purity standard required, as purity generally decreases with each subsequent generation of propagation. Hence, the seed production programmes have to be strictly monitored to ensure genetically pure and quality seed. In Tamil Nadu, more than 15 paddy varieties are being grown predominantly by the farmers and maintaining the seed genetic purity largely relies upon GOT (Grow Out Test) which is time taking and laborious. Moreover, characterization based on morphological markers was usually influenced by macro and micro environment, plant age, the genetic makeup of seed, environment and field management practices (Singh and Rachie, 1985) [12]. Thus the morphological descriptors reflected not only the genetic constitution of cultivar but also interaction of genotype and environment Ravi (2000) [10]. Li *et al*. (2002) [6] reported that because of environmental effects, diversity analysis based on phenotypic values may not be a perfect representation of the natural groupings of cultivars.

This necessitated the need for using molecular markers in distinguishing crop varieties as well as identification of seed lots with admixtures. So, it is necessary to employ suitable genetic models or molecular markers such as SSR or AFLP to reduce environmental effects or experimental errors and allow the quantitative prediction of genotype values of the crop. Because of their co-dominant expression and multi allelism microsatellite markers exhibited higher PIC values (Ferreira and Grattapaglia 1998) [5] which had promoted the application of microsatellites as molecular markers in fingerprinting (Ashikawa *et al*., 1999) [2] of crop varieties. In this context, use of molecular markers will help us in identifying seed lots with admixtures.

Indian Minimum Seed Certification Standard (1988) recommends a sample size of 800 seeds for foundation seed and 400 seeds for certified seed for assessment of seed genetic purity. DNA finger printing approaches based on PCR (Polymerase Chain Reaction) is being recommended by Government of India for seed genetic purity analysis in the Seed Testing Laboratory. However, assessment of seed genetic purity using molecular markers on individual seed basis is very difficult for 400 or 800 seeds. Hence an attempt was made to standardize the molecular seed genetic purity testing method by pooling of seeds. In this study seed samples of two varieties were pooled in four different ratios i.e. 9:1, 24:1, 49:1 and 99:1 to identify the threshold limit seed contamination that can be detected using SSR markers. Both the markers were able to discriminate the seeds of two varieties mixed in the ratio of 9:1 as well as in the ratio of 24:1 whereas seeds mixed in the ratio of 49:1 as well as 99:1 were not detectable by SSR markers showing a threshold limit of 5% admixtures to be detected by this molecular method.

The results of this study involving popular varieties Bhavani admixed with CO 43 and ADT 37 admixed with ASD16 genotyped with polymorphic SSR markers namely RM587 and RM1880 indicated that the optimum ratio for detecting the offtypes was 24:1 (Fig. 1). This might be due to the reduction in concentration of offtype DNA as the ratio increases. As per Indian Minimum Seed Certification Standard (1988), the seed lot can be rejected if the contamination is > 1% and 2% for foundation and certified seeds respectively. The efficiency of the method can further be improved by increasing the DNA concentration to 100ng per PCR reaction and increasing the

number of PCR cycles so that more amount of template DNA of admixtures are available to be detected by PCR analysis. Further to confirm the percentage of purity, the batch which showed the offtype has to be tested again on individual plant basis. Thus this method would help in replacing the present day GOT by reducing the time, space, labour and especially the environmental influence.

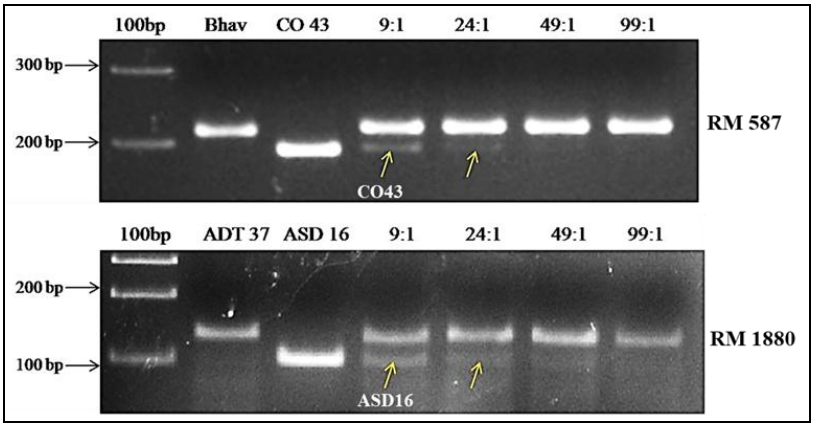

Fig 1: PCR analysis using DNA isolated from seed mixture of rice varieties in different ratios indicates the contaminants in 9:1 and 24:1 ratios can be detected through SSR markers.

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