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## Research Article

### Application of SSR marker in genetic purity analysis of CORH 4 rice hybrid and its parental lines

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#### Abstract

Grow out test is used for assessing the genetic purity but molecular markers like simple sequence repeats can be used as alternative. The present study aims at evaluating the efficacy of SSR marker in genetic purity assessment. SSR marker RM570 which differentiate the CMS 23A and CB 174R lines with the allelic size of 260 and 300 bp respectively. Four hundred paddy seeds comprising 2 off type and 398 original seeds of A, R and CORH 4 were sown in 20rows×20columns matrix in a sand medium and leaf samples from seedlings were bulked row and column wise. RM570 produced single allele for genetically pure bulking and two alleles for bulking with off types. Further, the identified off-types were investigated by individual leaf analysis using RM570 marker. Hence, SSR markers can be employed to the assessment of genetic purity rather than conventional grow out test.

#### Keywords

Rice hybrids, Simple Sequence Repeats, Genetic purity, Grow Out Test

#### INTRODUCTION

Agriculture plays a key role in the Indian economy, as more than 50 per cent of the population depends directly on agriculture and allied sector (Chand *et al.*, 2007). Increasing population accompanied by fragmentation of land holding down through the generation demands acceleration in food grain production. Amid a wide variety of cultivated crops wheat, rice and maize are of the first magnitude as they are the staple food for the majority of the world population. More than 90 per cent of the population in Asia depends on rice for meeting their diurnal dietary requirements (Bora *et al.*, 2016; Challa and Kole., 2019). Most of the conventional and agronomic approaches for enhancing the yield have hit the rock bottom. Therefore, development and utilization of high yielding varieties and hybrids is the key to feed the hungry mouths which can be achieved only by doubling the farmer's income (Bhaskar and Umarani, 2016).

Discovery of male sterility paved way for utilization of heterosis or hybrid vigour in the crop plants which emanated from the science of hybrid seed production.

In India, CGMS (Cytoplasmic Genetic Male Sterility) otherwise, known as 3-line system, consisting of A (male sterile line), B (maintainer line) and R (restorer line) line is widely used for hybrid seed production. (Nandakumar *et al.*, 2004; Waza *et al.*, 2013). Hybrid seed production is a skilled program, which is hindered by the presence of pollen shedders in the male sterile line, outcrossing with the alien pollen and physical admixtures which leads to deterioration of genetic purity (Nandakumar *et al.*, 2004; Waza *et al.*, 2013, Kumar *et al.*, 2012). It has been estimated that for every 1 per cent impurity in hybrid seed reduces the yield by 100 kg/ha (Mao *et al.*, 1996). Therefore, maintenance and assessment of genetic purity of the hybrid seeds are extremely important (Nataraj *et al.*, 2016 and Lu *et al.*, 2018).

Genetic purity test is the process of assessing the genuineness of the variety or hybrid. For seed certification assessment of genetic purity for the hybrids is compulsory. Conventionally, it is assessed through Grow Out Test (GOT)

(Cooke, 1995 and Tamilkumar *et al.*, 2009), that mainly depend on the plant morphological traits from seedling stage to harvest for detection of off-type in the population. Majority of the key differentiating plant traits are influenced by the environment (Kumar *et al.*, 2012 and Bora *et al.*, 2016). GOT is a slow, labour intensive and tedious and high cost involving task besides misleading the seed officers (Waza *et al.*, 2013). To succeed in dealing with the problem molecular assays with DNA markers have been utilized as they are precise, swift, reproducible and reliable. Microsatellite in general and Simple Sequence Repeats (SSR) in particular are extensively employed in fingerprinting, marker assisted selection and assessment of seed purity. The present paper describes the efficacy of the SSR marker in the assessment of genetic purity of CORH4 rice hybrid and its parental lines.

## MATERIALS AND METHODS

The present study was carried out at the Department of Seed Science and Technology, Tamil Nadu Agricultural University (TNAU), Coimbatore. Genetically pure F<sub>1</sub> seeds of CORH 4 and their parental lines CMS 23A (male sterile line), CMS 23B (maintainer line) and CB 174R (restorer line) were obtained from Paddy Breeding Station, TNAU, Coimbatore.

### Collection of plant samples and genomic DNA preparation

The DNA was isolated from the healthy young leaf samples using the CTAB method using homogenized autoclaved pestle and mortar (Beek *et al.*, 1992). The precipitated DNA was dissolved in TE buffer and checked for quantity and quality using Nano photometer analyser. Furthermore, the concentration of DNA was optimised to 100ng/μl and stored at -20°C until future use.

### DNA amplification

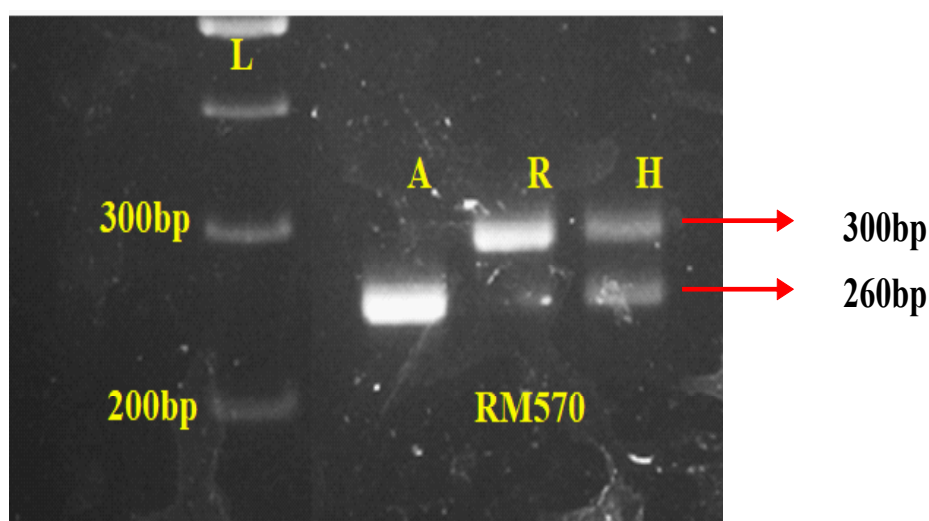
Twenty SSR markers were analysed and RM570 (F-GTTCTTCAACTCCCAGTGCG and R-GTTCTTCAACTCCCAGTGCG sequence) exhibited polymorphism between the male sterile and the restorer line of CORH 4 hybrid (Fig. 1). Therefore, it was utilized for assessing the genetic purity.

### PCR condition

The DNA was subjected to polymerase chain reaction in a total volume of 10 μl that contained 200ng/μl of template DNA, 6 μl of master mix, 0.5 μl of forward and reverse primer each. Thermal profile for SSRs was 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and ending up with 10 min at 72°C for the final extension. The amplification products of SSR markers were resolved in 3 % agarose gel at 120 V for 3 hours and the gel image was documented using the gel documentation unit.

### Genetic purity analysis

To assess the genetic purity, 398 seeds were randomly picked from each seed lots of CMS 23A, CB 174R and CORH 4 and 2 off-type seeds were introduced as a contaminant. The seeds were sown in 20 rows × 20 columns grow out matrix (Kumar *et al.*, 2012) in a sterilized sand medium and kept at 25°C and 95 ±3% RH in germination room. Leaf samples were collected separately from twenty-one days old individual seedlings and bulked row and column wise such that a total of 40 bulked leaf samples (20 each from row and column) are obtained from A and R lines separately. But for CORH 4 hybrid randomly selected 20 leaf samples were used for analysis. DNA was extracted and amplified using the marker RM570 for detection of off type seedling.



**Fig. 1. Molecular profile of CORH 4 hybrid and its parental lines obtained with RM570. L=100bp Ladder, A= CMS 23A, R=CB 174R, H=CORH 4.**

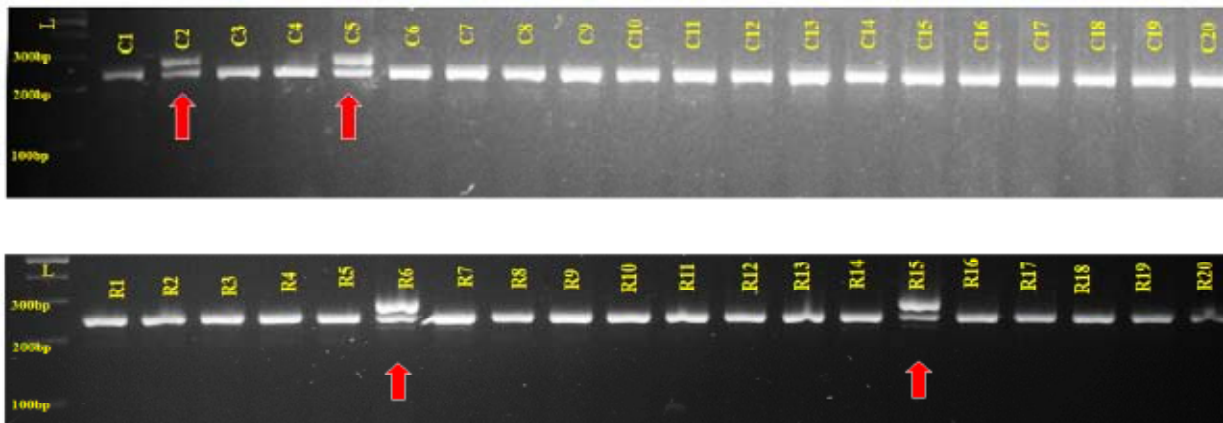
Percentage of off-types in the seed lots can be assessed by the given formula.

Per cent off-types =

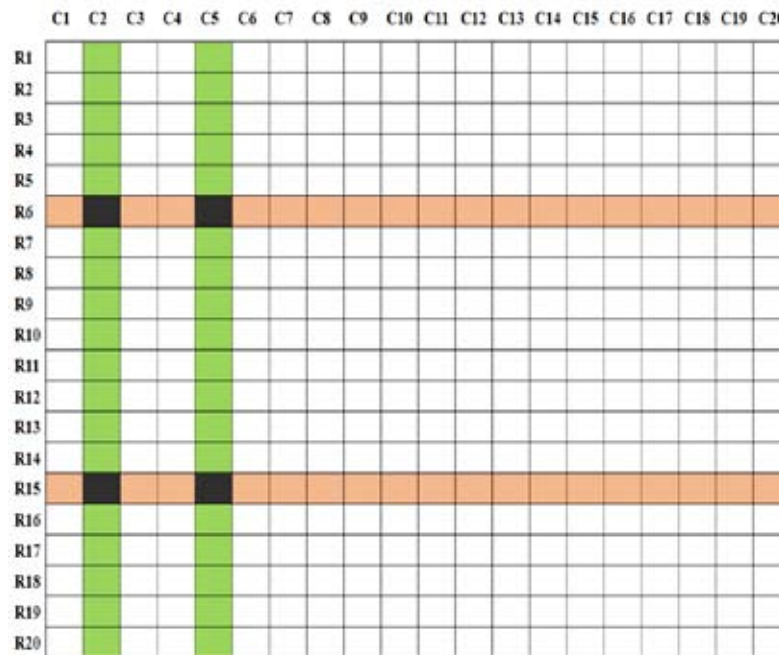
$$\frac{\text{Total number of off-type seeds}}{\text{Total number of seeds sown}} \times 100$$

**RESULT AND DISCUSSION**

Assessment of genetic purity of seeds is crucial for maintaining the seed quality in paddy hybrid seed production. Among the twenty SSR markers RM570 differentiated the CMS 23A and CB 174R lines with the allelic size of 260 and 300 bp respectively. Thus, ensuring that the allele sizes are specific to the seed and pollen parent of CORH 4 hybrid. Furthermore, hybrid exhibited both alleles of size 260 and 300 bp as expected, which confirmed the genuine crossing and heterozygotic condition (Fig.1)



**Fig. 2a.** Assessment of Genetic purity of CMS 23A line using SSR marker RM570. L=100bp Ladder, Lane C1-C20 = Column wise bulked samples, Lane R1-R20 = Row wise bulked samples, Lane C2, C5, R6 and R11 = Off-types or contaminants.



**Fig. 2b.** Diagrammatic representation of the 20 rows × 20 columns of CMA 23A with the coordinates (black coloured) indicating possible contaminants.

In order to test the purity of CMS 23A line the PCR products of row and column bulked samples were resolved on the agarose gel which showed amplification of both the alleles i.e., 260 and 300 bp (double band) at 2<sup>nd</sup>, 5<sup>th</sup> and 6<sup>th</sup>, 15<sup>th</sup> lanes of column and row bulked samples respectively (Fig. 2a). It was an indication that the contaminant might be present on either C2R6, C5R15 or C2R15, C5R6 coordinates (Fig. 2b). As we have added only two off-type

seeds it was unlikely that all four coordinates could be off-types. In order to detect the actual off-types individual leaf samples from seedlings at four coordinates were analyzed. The results revealed that allele size of seedling at the coordinates C2R15 and C5R6 was 260 bp similar to that of CMS 23A but at coordinates, C2R6 and C5R15 the allele sizes were both 260 and 300 bp which confirmed them as the off-type seedlings (Fig. 5).

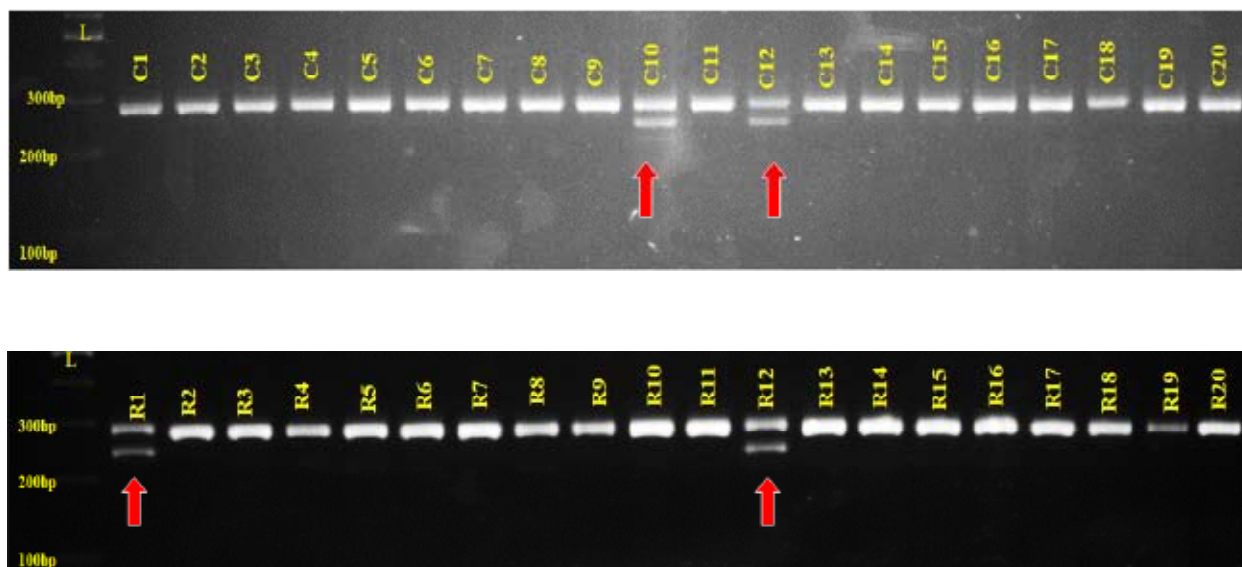


Fig. 3a. Assessment of Genetic purity of CB 174R line using SSR marker RM570. L=100bp Ladder, Lane C1-C20 = Column wise bulked samples, Lane R1-R20 = Row wise bulked samples, Lane C10, C12, R1 and R12 = Off-types or contaminants.

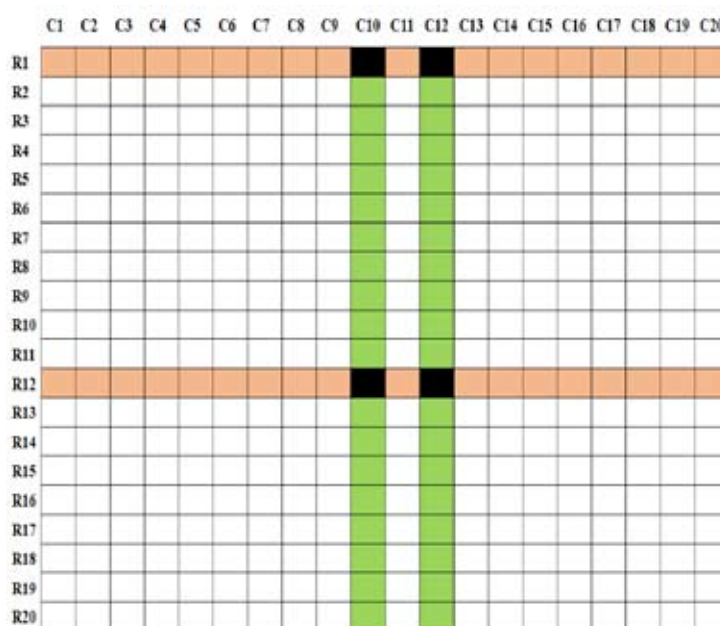
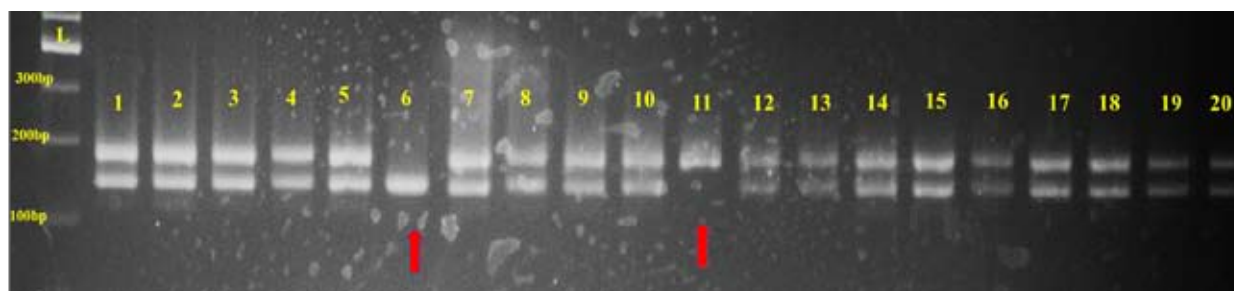
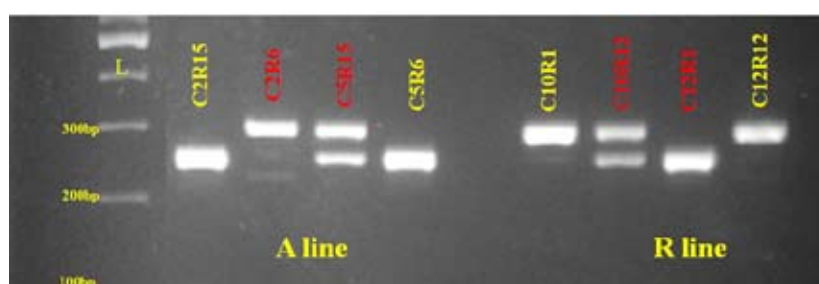


Fig. 3b. Diagrammatic representation of the 20 rows x 20 columns of CB 174R with the coordinates (black coloured) indicating possible contaminants.



**Fig. 4. Assessment of Genetic purity of CORH 4 hybrid using SSR marker RM570. L=100bp Ladder, Lane 1-20 = Individual hybrid plants, Lane 6 and 11 = Off-types or contaminants.**



**Fig. 5. Off-type confirmation among the four suspected coordinates of A line and R line. L=100bp Ladder, A line = CMS 23A, R line = CB 174R, Yellow coloured = True to Type, Red coloured = Off-types or contaminants.**

Genetic purity test of CB 174R line showed that 10<sup>th</sup>, 12<sup>th</sup> and 1<sup>st</sup>, 12<sup>th</sup> lane of gel exhibited amplification of both the alleles i.e., 300 and 260 bp (double bond) for column and row bulked samples respectively (Fig. 3a). It was a hint that the contaminant might either be present on C10R1, C12R12 or C10R12, C12R1 coordinates (Fig. 3b). As we have added only two contaminant seeds it was unlikely that all four coordinates could be off-types. In order to confirm the off-types individual leaf samples from seedlings at four coordinates were analyzed. The results revealed that allele size of seedling at the coordinates C10R1 and C12R12 were 300 bp as that of CB 174R but at coordinates, C10R12 and C12R1 the allele sizes both were both 300 and 260 bp which confirmed the off-type seedlings (Fig. 5).

To test the genetic purity of CORH 4 hybrid, twenty seedlings were analyzed which exhibited both the alleles i.e., 260 and 300 bp (double bond) except 6<sup>th</sup> and 11<sup>th</sup> seedling which amplified allele sizes of 260 and 300bp respectively (Fig. 4) which confirmed them to be the off-type seedlings. Furthermore, the per cent of off-types

are depicted in **Table 1** calculated using the formula mentioned above.

Hybrid seed production is a skilled technique which involves both maintenance of male sterility and production of hybrid which has few lacunae like outcrossing, crossing with undesired foreign pollen and mechanical admixtures during harvesting and post-harvest stages. These admixtures act as a source of pollen shedders or off-types in the seed production plots. Hence, the grow out test is conducted to assess the genetic purity which is time consuming and an alternate to assess the purity would be through molecular markers. Previous studies conducted in rice (Nandakumar *et al.*, 2004; Tamilkumar *et al.*, 2009 Sudharani *et al.*, 2013 and Nataraj *et al.*, 2016), maize (Daniel *et al.*, 2012) and brinjal (Pattanaik *et al.*, 2018) confirms the feasibility of purity assessment by employing varietal or hybrid-specific SSR marker. This technique is accurate, rapid, reliable cost cutting and reproducible. Hence, it can be used by seed analysts, seed producers and breeders for testing the genetic purity of the seed samples.

**Table 1. Percentage of off-types in the male sterile, restorer line and hybrid**

Line	No of off-types	Off-type per cent	Genetic purity per cent
CMS 23A	2	0.5	99.5
CB 174R	2	0.5	99.5
CORH 4	2	0.5	99.5

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