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Validation of reported markers for seed dormancy and pre-harvest sprouting resistance in rice (*Oryza sativa*. L)

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Abstract
DNA markers have enormous potential to improve efficiency and precision of conventional plant breeding *via* marker assisted selection. To test the usefulness of microsatellite markers associated with pre-harvest sprouting resistance QTLs in rice, 32 diverse rice genotypes and two F_2 populations were used. A total of 24 reported SSR markers were used to reveal polymorphism between dormant and non-dormant rice genotypes. Among the 24 tested SSR primers five markers *viz.*, RM252, RM17, RM564, RM480 and RM346 showed polymorphism for pre-harvest sprouting resistance among 32 genotypes. The phenotyping results revealed that MTU 1075, MTU 3626, MTU 5293, MTU 1010, BPT 2658, BPT 2741, BPT 2411, RGL 1414 and BPT 3291 possess moderate dormancy for 12 DAH while MTU 1001 exhibited strong dormancy. The phenotypic germination patterns of 32 genotypes at different intervals were compared with the genotyping results using Map Disto method of analysis. The test of goodness of fit χ^2 test was conducted for phenotypic germination patterns of 32 genotypes and genotypic data developed by five polymorphic markers and the results revealed that two markers *i.e.*, RM252 and RM17 exhibited significance. Among the 6 markers studied, the primers RM21 and RM252 were able to show distinct polymorphism between dormant and non-dormant genotypes among the individuals selected from BPT 5204/ MTU 1001 F_2 population while RM480 and RM235 showed a good characteristic polymorphism among the selected individuals of BPT 2231/MTU 1001 F_2 population.

Keywords: Validation, markers, seed dormancy, rice

Introduction
Rice is the main staple food crop for majority of global population. In India, rice is cultivated over an area of 43.86 M ha with a production of 105.48 Mt and productivity of 2424 kg ha^{-1} (India stat, 2014-15) [9]. Yield and quality enhancement are the major breeding objectives in rice improvement programmes. One of the most important agronomic problems in rice production in high humid climates is pre-harvest sprouting (PHS) which is a phenomenon that is responsible for large losses to agricultural industry particularly in coastal areas in some years. Screening of rice germplasm possessing the genes for PHS resistance with molecular markers and exploitation of these markers will help the plant breeders in choosing suitable parents for their hybridization programmes. The pre-harvest sprouted grain looses it's viability rapidly during storage (Moor, 1987) [10]. Even mild levels of sprouting damage can substantially reduce grain storage life (Bason *et al.*, 1991) [2]. The long term solution to pre-harvest sprouting relies on the development of cultivars that are able to tolerate the damaging effects of rain during the period between maturity and the completion of harvest. As this trait is highly influenced by climatic factors, screening of cultivars and selection on the basis of phenotype is problematic. Therefore, molecular markers linked to genes associated with pre-harvest sprouting resistance could be a useful tool in marker assisted selection in order to develop the cultivars with resistance to pre-harvest sprouting.

Materials and methods
A. Plant materials
The experimental material comprising of thirty two genotypes obtained from Rice Research Unit (RRU) Bapatla, RARS, Maruteru and two F_2 populations from *viz.*, BPT2231/MTU1001 & BPT 5204/MTU 1001. The F_1 plants were raised during *kharif* 2015 and the resulting F_2 seed along with 32 genotypes were sown during *Rabi* 2015-16 to obtain F_2 population for genotyping with reported SSR markers. A total of 94 individuals each from two F_2 populations were screened for pre-harvest sprouting resistance. Phenotyping studies for all the 32 genotypes and F_2 populations were carried out at Rice Research Unit,

Bapatla while the genotyping was done at Indian Institute of Rice Research (IIRR), Hyderabad.

B. Phenotypic screening of dormancy

The dormancy was evaluated in terms of percentage of germinated seeds and dormancy was assessed following the procedure described by Ikehashi (1972) [6] and Wan *et al.* (1997) [14]. Panicles from randomly selected plants in each replication were collected on the 35th day after heading and these seeds were subjected to heat treatment at 50ºC for 3 days in an oven. For evaluation of dormancy, 100 seeds were counted and were placed in moist filter papers which are plated in petriplates with size of 15 cm diameter and kept in incubator at 30ºC and then germination counts were taken on 7th day and 10th day after soaking. Those seeds with a coleoptile longer than 2mm was counted as germinated. These germination tests were conducted on each genotype for five times at different intervals starting from 3 days after harvesting (3DAH) followed by 5DAH, 7DAH, 10DAH and 12DAH.The intensity of dormancy was assessed for all genotypes under study and were categorised as weakly dormant (>80%), moderately dormant (50-79%) and strongly dormant (<50%). The time taken for attaining 80% germination after maturity was considered as the duration of dormancy (Voleti *et al.*, 2013) [13].

C. Genotyping for PHS resistance

Leaf samples of 32 genotypes along with F_2 population of two crosses were collected from 12 day old seedlings and the DNA was isolated using C-TAB method as described by Doyle and Doyle (1990) [4]. The quantification of the samples was done through the gel electrophoresis with 0.8% agarose gel. The genomic DNA of the selected lines subjected to PCR amplification as per the procedure described by Chen *et al.* (1997) [3].

A total of 24 reported SSR markers were used to reveal polymorphism between dormant and non-dormant rice genotypes. Among the 24 tested SSR primers, the markers which manifested clear polymorphism for both the parents in the parental polymorphic survey were utilized for genotyping of F_2 populations.

E. Data Analysis

For each of the defined loci, SSR allelic composition, allelic frequencies, alleles per locus was determined for each genotype. The PIC value was determined following the formula proposed by Anderson *et al*. (1993) [1]. The phenotyping and genotyping data obtained from the screening of 32 genotypes for validation of reported markers were analysed with Map Disto software to know the significant association of the polymorphic markers with the trait. Allelic segregation at each of the marker loci was analysed for deviation from the expected 1:1 ratio in the genotypes using λ^2 test of significance obtained from Map Disto version 1.7.5 software (Lorieux, 2007) [7]. Using the DARWIN version 6 software (http://darwin.cirad.fr/) (Perrier *et al.*, 2005) [11], a simple matching dissimilarity index was calculated from the allele-size data set (single allele data) and this matrix was then subjected to Neighbour-Joining analysis to construct the dendrogram.

Results and discussion

1. Phenotyping of genotypes

The results of phenotyping of 32 rice genotypes for seed dormancy revealed that BPT 5204 and BPT 2231 exhibited > 80% germination at 5 days after harvest (DAH) suggesting that they are weakly dormant. The genotypes *viz.*, MTU 1075, MTU 3626, MTU 5293, MTU 1010, BPT 2658, BPT 2741, BPT 2411, RGL 1414 and BPT 3291 manifested < 10% germination at 5DAH and < 50% germination even after 12DAH suggesting that these genotypes are moderately dormant up to 12 DAH. Among the genotypes studied, MTU 1001 recorded < 10% germination even after 12DAH suggesting that this genotype possessed strong seed dormancy. The cultivars with less germination percentage at maturity (<5%) and at the end of one week after maturity (10%) should be preferred for cultivation in the coastal regions during *kharif* season, hence it may be concluded that MTU 1001, MTU 1064, MTU 1010, MTU 5249, BPT 2741 and BPT 2658 may be recommended for cultivation in coastal areas.

2. SSR polymorphism

To test the usefulness of markers, 24 SSR markers reported in public domain present on different chromosomes were used to reveal molecular diversity for pre-harvest sprouting resistance. Among the 24 SSR primers, 19 primers showed monomorphic banding pattern while 5 markers *viz.*, RM252, RM17, RM564, RM480 and RM346, showed considerable polymorphism. The number of alleles detected per primer ranged from 2 (RM17, RM564, RM480 and RM346) to 3 (RM252). The Polymorphic Information Content (PIC) value calculated to estimate the in formativeness of each primer varied from 0.67 in RM480 to 0.79 in RM252 with an average of 0.73 thus indicating that all five primers were capable of distinguishing between genotypes and highly informative. The markers RM17, RM564, RM480 and RM346 recorded the PIC values of 0.72, 0.78, 0.67 and 0.78 respectively (Table 1). These results revealed that the marker RM252 would be best in screening rice germplasm for PHS resistance on chromosome 4 followed by RM564, RM346 and RM17.

Table 1: Allele, PIC value of the markers that shown polymorphism for PHS resistance

Marker	alleles	PIC
RM 17	2	0.72
RM564	2	0.78
RM480	2	0.67
RM346	2	0.78
RM252	3	0.79

The amplification pattern with the primer RM 17 on chromosome 12 manifested a clear polymorphism among the tested material. Out of 32 genotypes under study, the bands amplified by six genotypes *viz.*, MTU 1001, MTU 1064, MTU1061, BPT 2658, BPT 2741 and RGL 1414 recorded similar amplicon size and differed with the banding patterns manifested by the remaining genotypes while the amplification pattern exhibited by four genotypes *viz.*, MTU 1001, BPT 1768, RGL 1414 and BPT 2673 was similar with RM 346 suggesting the presence of similar QTLs with small effects in these genotypes. The results of genotypic screening with 24 SSR markers revealed that the variety, MTU 1001 manifested similar amplification pattern with all the five markers which exhibited polymorphism suggesting the presence of several QTLs on chromosomes 4, 12, 5, 6 and 7 governing the seed dormancy which all together contributed to the pre-harvest sprouting resistance. The results of

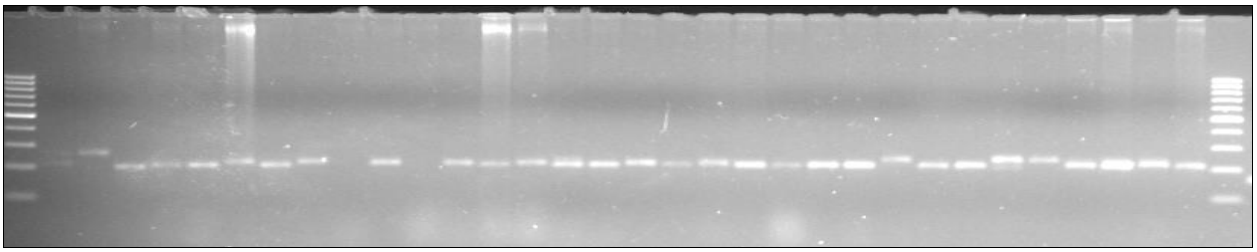
phenotypic data also indicated that MTU 1001 exhibited only <10% germination even after 12 days after harvesting which is a desirable trait that should be incorporated in other varieties cultivated in coastal regions. Previously, Voleti *et al.* (2013) [13] also reported similar findings of seed dormancy in MTU 1001 for 9 weeks after harvesting. Hence, this variety can be utilized in the crossing programmes for identification of QTLS controlling PHS further these QTLs may be transferred into any susceptible varieties using MAS.

The genotyping results of five markers *viz.,* RM252, RM17, RM564, RM480 and RM346 which showed polymorphism for PHS resistance among 32 genotypes were compared with the phenotypic germination patterns of 32 genotypes at different intervals using Map Disto method of analysis. The test of goodness of fit (λ^2 test) was conducted for phenotypic data of 32 genotypes and genotypic data developed by five polymorphic markers (Table 2). The results revealed that out of five markers which manifested considerable polymorphism among genotypes tested, two markers *i.e.,* RM252 and RM17 only exhibited significance. Hence, it may be concluded that the phenotypic data generated for 32 genotypes was in accordance with the genotypic data manifested by RM252 and RM17 markers suggesting that these two markers may be utilized for genotyping of seed dormancy in rice to improve the selection efficiency for PHS resistance.

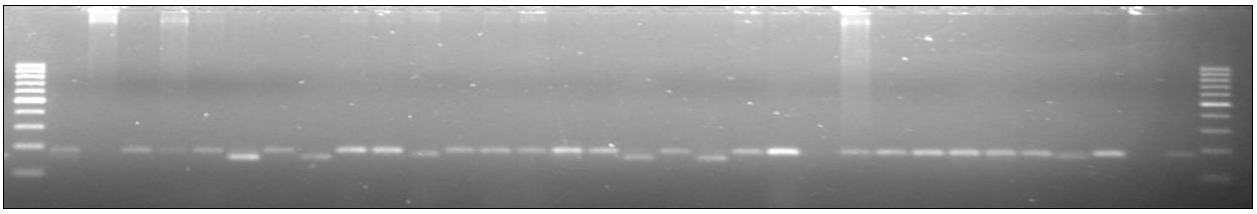
Table 2: Test of goodness of fit (λ^2) for five polymorphic markers obtained from Map Disto analysis

N°	Marker	$\lambda^2_{1:1}$	*P*
1	RM 252	10.80	0.00102**
2	RM 17	9.97	0.00160**
3	RM 564	2.29	0.13057 (ns)
4	RM 480	0.08	0.78151 (ns)
5	RM 346	2.79	0.09467 (ns)

**significance at 1% level.



Amplification profile of the DNA of 32 rice genotypes using the primer RM252.



Amplification profile of the DNA of 32 rice genotypes using the primer RM17

3. Cluster Analysis

Cluster analysis was done using DARWIN version 6 software. Dendrogram obtained from analysis grouped the 32 genotypes into two major clusters and one minor cluster (Fig 1). The major clusters are A and B. Cluster A is again divided into A1 and A2. Subcluster A1 contains 12 genotypes and 4 genotypes were clustered into A2. Similarly, cluster B is again sub clustered into B1 and B2. B1 contains 8 genotypes and B2 contains single genotype whereas, the cluster C contains 7 genotypes. Majority of the genotypes grouped in cluster B possess PHS resistance and contains the QTLs linked to marker RM252 and RM17. Majority of the genotypes grouped in cluster C showed positive amplification for the marker RM564 which is linked to the QTL possess QTLs linked to RM564 marker.

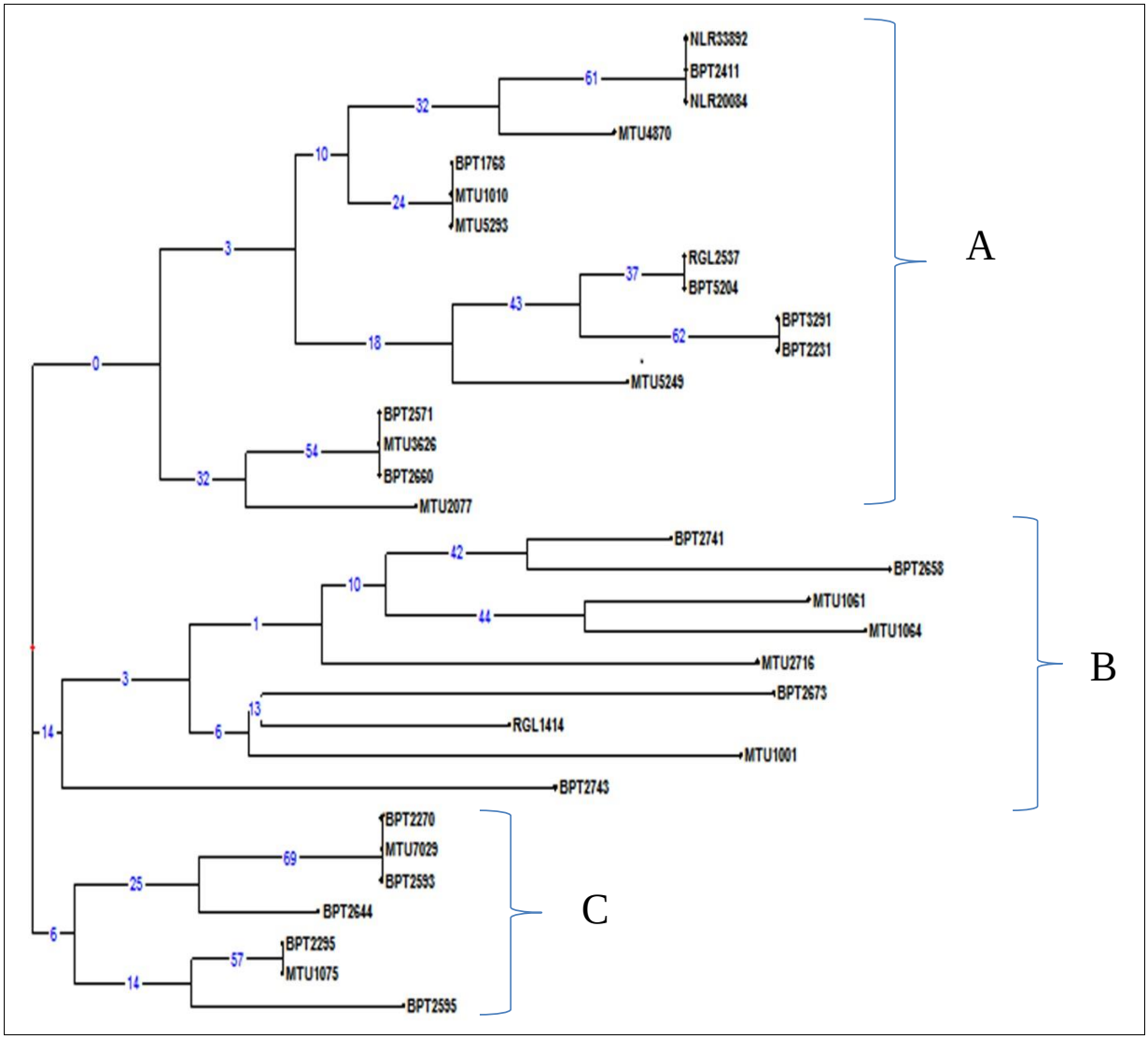


Fig 1: Dendrogram showing genetic relationship among 32 rice genotypes based on pre-harvest sprouting resistance polymorphic SSR markers derived from UPGMA cluster analysis using DARWIN software.

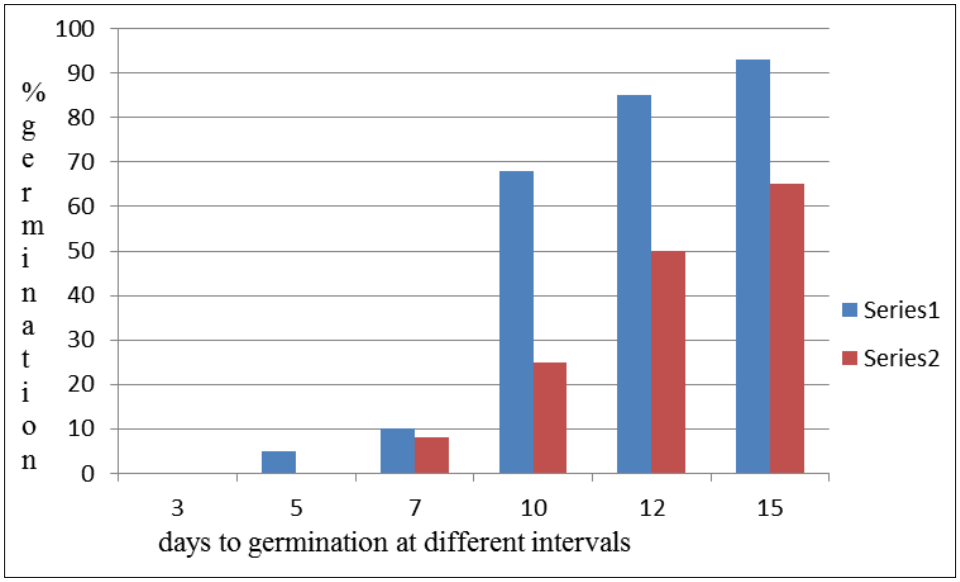
4) Screening of F_2 Populations

A total of 94 individuals each from both F_2 populations were screened with reported makers for dormancy. A total of 6 markers which manifested clear polymorphism for both the parents in the parental polymorphic survey were utilized for genotyping of F_2 populations. Among the 6 markers studied, the primers RM21 and RM252 were able to show a distinct polymorphism among the individuals from BPT 5204/ MTU 1001 F_2 population. Gu *et al.* (2005) [5] also used RM252 for detection of QTLs for seed dormancy on chromosome 4 from EM93-1/SS18-2 population. Likewise, the use of RM21 for dormancy studies was earlier reported by Rathi *et al.* (2011) [12] while deciphering the location of QTL on chromosome 11 using F_2 population of the cross combination of Cheni ahu (dormant) and Kolong (non dormant) parents.

Ninty four individuals from the F_2 populations of BPT 2231/MTU 1001 were selected for performing PCR along with the parents using six primers but two primers *i.e.*, RM480 and RM235 showed polymorphism. Lu *et al.* (2011) [8] also used these markers located on chromosome 5 to study the genotyping of using F_2 population of two cross combinations *i.e.*, Q4359/Nanjing 35 and Q4646/ Nanjing 35.

Based on the results of the molecular marker studies conducted on two F_2 populations and also among 32 genotypes it may be concluded that the markers RM21, RM17, RM252, RM480 and RM235 showed considerable polymorphism among dormant and non-dormant individuals and can be utilised for screening of PHS resistance in rice. Further, the markers, RM21, RM252 and RM480 showed their utility in marker assisted selection for proper identification of individuals possessing PHS resistance in early generation segregants.

The phenotyping data *i.e* the germination patterns of the F_2 populations (Table 3) has shown the continuous variation and is shown in the graph (Fig 2) for both the populations when drawn with the germination percentages at different classes of intervals against the no. of days taken for sprouting at regular intervals of time. The graph has skewed towards the mean germination percentage of the susceptible parent showing that the no. of segregants in the population are having the limited number of individuals with resistance to PHS from the selected population. The continuous variation in the population suggested that the trait under study may be governed by many polygenes.



Series 1: F_2 population of BPT 5204/ MTU1001
Series 2: F_2 population of BPT 2231/ MTU 1001

Fig 2: Distribution of preharvest sprouting percentage in F_2 population of two populations.

Table 3: Germination frequency of F_2 populations at different days after harvest

Days of interval	Germination Percentage of BPT 5204/ MTU1001	Germination Percentage of BPT 2231/ MTU 1001
3 DAH	0	0
5DAH	5	0
7DAH	10	8
10 DAH	68	25
12 DAH	85	50
15 DAH	93	65

Conclusion

Based on the results of the molecular marker studies conducted on two F_2 populations and also among 32 genotypes it may be concluded that the markers RM21, RM17, RM252, RM480 and RM235 showed considerable polymorphism among dormant and non-dormant individuals and can be utilised for screening of PHS resistance in rice. Further, the markers, RM21, RM252 and RM480 also showed their utility in marker assisted selection for proper identification of individuals possessing PHS resistance in early generation segregants.

Both phenotyping and genotyping results among 32 genotypes in the present study revealed that the variety MTU 1001 with <10% germination exhibited strong PHS resistance and it might have possessed different QTLs on chromosomes 4, 5, 6, 7 and 12. Likewise, QTLs linked with RM17 and RM252 markers on chromosomes 4 and 12 respectively were present in MTU1061, MTU1064 and BPT2411. Similar amplification pattern withRM564 and RM346 for BPT 2295, BPT 2673 and RGL 1414 indicated that QTLs linked to these two markers may be present in these genotypes.

The phenotypic variation in the segregation patterns of the two populations has shown the continuous distribution of individuals that clearly depicts that the trait is governed by polygenes so the variation in the population is continuous showing quantitative nature of the PHS trait.

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