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Study of diversity among rice accessions with varied level of Brown plant hopper resistance using molecular markers

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Abstract
The brown planthopper (BPH), *Nilaparvata lugens* Stal (Homoptera: Delphacidae), is the most-significant insect pest of rice (*Oryza sativa* L.) throughout rice-growing countries. In this study DNA-based SSR molecular marker technique was deployed to estimate genetic diversity among 30 rice accessions using seven SSR primers which produced a total of 32 polymorphic markers with the average number of 4.57 markers per primer. The PIC value for all 7 microsatellite loci in the present study ranged from 0.500-0.833. The PIC value was maximum in case of RM186 (0.833) and all the SSR markers were more informative since they recorded more than 0.500 as PIC value. The seven primer pairs showed 3-6 alleles across 30 genotypes grouping the genotypes into 7 groups of different composition indicating that even seven SSR primer pairs can differentiate the cultivars. The analysis indicated that co efficient of similarity among 30 varieties ranged from 0.67 to 1. In the present study, SSR markers were able to reveal greater genetic diversity among the rice varieties and SSR locus RM217 on chromosome # 6 was found to be putatively associated with BPH resistance.

Keywords: rice, *Nilaparvata lugens*, simple sequence repeats

Introduction
Rice (*Oryza sativa* L.), is the staple food for more than half of the world population [1]. A number of biotic and abiotic stresses limit the rice production in many tropical regions. The brown planthopper (BPH) *Nilaparvata lugens* is the most destructive insect pest in Asia [2]. This monophagous pest is a major threat to rice production by sap-sucking and by acting as a vector of rice stripe virus, rice grassy stunt virus and ragged stunt virus, which can cause even more serious yield reduction.

Microsatellites, which are also called simple sequence repeats (SSRs), are short tandem repetitive DNA sequences with a repeat length of a few (1-5) base pairs. SSR markers are co-dominant molecular markers and are widely used in rice genetic analyses, genome mapping and marker-assisted breeding. Because of their widespread distribution in the genome, SSRs have become a valuable source of genetic markers. Markers based on simple sequence repeats (SSR) have been shown to be highly polymorphic even between closely related individuals within a species [3] and tend to show more polymorphism than many alternative marker systems [4]. Molecular marker technique has opened the possibility for marker assisted selection and breeding using gene-tags, to evolve durably resistant cultivars in shorter span of time with greater accuracy.

The availability of molecular linkage maps in rice [5, 6] has facilitated the identification and easy manipulation of major genes and polygenes (Quantitative Trait Loci or QTL), conferring resistance to insects. Map-based cloning represents one possible approach to isolate BPH resistance genes and elucidating the BPH resistance mechanism in rice. To achieve map-based cloning, construction of a high-resolution linkage map with DNA markers is required. For that, selection of diverse parents is essential to develop mapping populations. With this background, the present study, is an attempt to explore the level of resistance to BPH in rice accessions and analyze their molecular diversity with DNA markers.

Materials and Methods
Plant materials
Mass rearing of brown plant hopper and seedling box screening rice cultivars for BPH resistance for 30 rice accessions were carried out as mentioned in our earlier report [7] (Vinod *et al.*, 2016). The plant materials used in this study included 30 accessions as screened in the

previous experiment. The experimental material consisted of 30 rice genotypes are CO43, IR50, ASD16, ADT36, PTB18, BASMATI 370, IR44, TN1, IR36, ASD7, IR32, TKM9, RATHU HEENATI, IR64, PTB33, W1263, PY3, TKM6, ASD18, PMK2, TKM10, ASD19, ADT37, AZUCENA, ADT41, IR26, IR38, MDU3, PTB20 and IR30

DNA extraction

Fresh leaf samples were used to extract DNA from the genotypes, following the method recommended by Gawel and Jarret (1991) [8] with slight modifications. Quality and quantity of isolated DNA was analyzed. Agarose gel electrophoresis was performed to check the quality of DNA.

DNA bulking of phenotypic extremes

DNA bulks, one for resistance class and other for susceptible class (measured by the level of resistance to BPH through screening experiments), were obtained by pooling the DNA of phenotypic extremes. The size of the individuals constituting each bulk was maintained as five. RATHU HEENATI, PTB33, ASD18, PTB18 and IR38 constituted the resistant bulks and CO43, TKM6, IR50, BASMATI370 and TN1 constituted the susceptible bulks.

PCR amplification using SSR primers

PCR analysis was done using SSR (simple sequence repeat) markers. A total of seven SSR primers were used for amplifying the genomic DNA. SSR primers used in this study were obtained from Sigma Aldrich Chemicals Private Limited, Bangalore, (India) and the sequence information is presented in Table 1. Amplification was carried out in 15 µL reaction volume containing 20-30 ng of genomic DNA, 0.8 µM of forward / reverse primer, 100 mM each of dATP, dGTP, dCTP, dTTP and 1.5 mM assay buffer. Amplification was done using a PTC Thermal Cycler (MJ Research Inc.) programmed for initial denaturation at 94ºC for 1 min, 30 cycles of 1 min. denaturation at 94ºC, 50 sec annealing at 46ºC and 2 min extension at 72ºC and final extension of 5 min at 72ºC and then at 4ºC for storage. Clearly resolved, polymorphic bands were scored visually. The score were obtained in the form of matrix with "1" and "0", which indicate the presence and absence of bands in each variety respectively.

Table 1: List of SSR primers used in the study and their sequences

SSR marker	1	2	3	4	5	6	Alleles	PIC Values
RM168	3	14	3	10			4	0.750
RM186	3	4	14	6	2	1	6	0.833
RM217	9	7	8	6			4	0.733
RM225	2	7	3	9	3	6	6	0.822
RM459	1	22	7				3	0.750
RM3331	2	2	2	6	10	8	6	0.821
RM261	2	28					2	0.500

Result and Discussion

In the present study, 30 rice varieties with varied level of resistance to BPH were evaluated for genetic diversity using simple sequence repeats (SSR) markers. All the 7 primers used in the study produced scorable, unambiguous markers. The SSR primers produced a total of 32 polymorphic markers across 30 rice varieties. The present study focused on assessment of genetic diversity among 30 rice varieties with varied classes of BPH resistance using SSR markers. The present SSR survey clearly detected high levels of polymorphism among the rice cultivars with multiple alleles identified at all 30 loci with an allelic range of 3-6. These numbers were consistent with the combined reports of Wu and Tanksley (1993) [9] and Prasad *et al*. (2000) [10] where in the allelic diversity of microsatellite markers in cultivated rice varieties were found to be in a range of 3-14 alleles per microsatellite locus. However, the number of SSR alleles were much smaller than those reported by Yang *et al*. (1994) [11] who observed an allelic range of 3-25 per SSR locus using different rice landraces and cultivars of both *japonica* and *indica* origin.

All the SSR primers produced polymorphic fragments. The bands generated in the rice varieties by SSR markers ranged from 3 (PTB 20 and IR 30) to 14 (W 1263). The PIC value was maximum in case of RM186 (0. 833) and all the SSR markers were more informative since they recorded more than 0.500 as PIC value (Table 2). PIC provides an estimate of discriminatory power of a locus by taking into account not only the number of alleles expressed, but also the relative frequency of those alleles. PIC values range from 0 (monomorphic) to 1 (very high discriminative) with many alleles in equal frequencies. All the SSR markers used were found to be highly informative in revealing the genetic diversity among the genotypes studied suggesting their potentiality in future genetic diversity analyses. The current investigation had a slightly higher average PIC value when compared to the reports of Olufowote *et al*. (1997) [12] who studied wide range of *indica* and *japonica* cultivars. The high PIC values obtained in the present investigation might be due to high genetic diversity among the varieties included, since the PIC values are dependent on the genetic diversity of the accessions chosen as reported by Garland *et al*. (1999) [13]. This study indicated the potential use of SSR marker to detect genetic variation.

The cluster analysis was used to group the varieties and to construct the dendrogram. A total of seven distinct groups were resulted out of the analysis for SSR (Fig.1). All the SSR markers obtained in the present study proved to be highly informative which was consistent with the results of Saghai-Maroof *et al*. (1994) [14] and Dje *et al*. (2000) [15] in sorghum and Ravi (2000) [16] in rice. The poly allelic nature of SSR markers has the advantage of discriminating the individuals more precisely. The seven primer pairs showed 3-6 alleles across 30 genotypes grouping the genotypes into 7 groups of different composition indicating that even seven SSR primer pairs can differentiate the cultivars. Highly informative SSR profiles can be generated among the rice cultivars by using as few as three primer sets [12]. Cluster analysis was carried out on two sets of marker profiling data based on SSR markers. SSR markers grouped the rice accessions into 7 clusters. The use of SSR markers resulted in the detection of 0.67 to 0.97 similarity coefficients for 30 rice accessions indicating the better efficiency of differentiating the varieties. In case of SSR maker analysis, IR 26, IR 38, ADT 41, AZUCENA formed the I cluster, IR 30, MDU 3 the II cluster, W 1263, PTB 20, ADT 37, PKM 2, ASD 18, PTB 33 the III cluster, TKM 6, IR 44, ASD 19, Basmati 370 the IV cluster, TN 1, IR 32, TKM 9 the V cluster, Rathu Heenati, IR 64, ASD 7, IR 50 the VI cluster, PY 3, IR 36, PTB 18, ADT 36, ASD 16, CO 43 formed the final cluster. The analysis indicated that co efficient of similarity among 30 varieties ranged from 0.67 to 1 (Fig. 1). The pools of TN 1, Basmati 370, IR 50, CO 43, TKM 6 and Rathu Heenati, PTB 33, ASD 18, ADT 37, IR 38 constituted the susceptible bulk and resistant bulk respectively. Among these primers, RM 217 on chromosome # 6 was found to show distinct difference between the

resistant and susceptible bulks. The marker profile of individual varieties those constituting the bulk are presented in Table 3. The efficiency of SSR markers in varietal differentiation and the availability of linked SSR loci associated with BPH resistance prompted to use the allelic distribution of SSR locus *viz.*, RM217 on chromosome # 6 to distinguish the resistant and susceptible varieties to BPH (Table16). This SSR locus was selected since there were reports indicating the detection of BPH resistance [2, 17]. Association analysis was done by bulking the samples from rice varieties having extreme phenotypes to link the microsatellite markers associated to brown plant hopper resistance genes / QTL. Seven primer pair was tried and RM 217 was found to be putatively linked. Similar results were obtained in earlier studies by Yang *et al.* (2002) [18] and Renganayaki *et al.* (2002) [19]. DNA fingerprinting should be considered a wholesome approach for studying the genetic variability of rice cultivars as it has great potential for practical use in resistance plant breeding.

Table 2: Allele distribution and PIC values revealed by SSR markers in rice varieties

Primers	Forward Sequence (5' to 3')	Reverse Sequence (5' to 3')	Rice Chr.# in which BPH locus was mapped earlier
RM 168	TGCTGCTTGCCTGCTTCCTTT	GAAACGAATCAATCCACGGC	3
RM 186	TCCTCCATCTCCTCCGCTCCCG	GGGCGTGGTGGCCTTCTTCGTC	3
RM 217	ATCGCAGCAATGCCTCGT	GGGTGTGAACAAAGACAC	6
RM 225	TGCCCATATGGTCTGGATG	GAAAGTGGATCAGGAAGGC	6
RM 261	CTACTTCTCCCCTTGTGTCG	TGTACCATCGCCAAATCTCC	4
RM 459	CTGCAATGCTGCATGACC	CACTTTCTCTGCAGCACCAG	5
RM3331	CCTCCTCCATGAGCTAATGC	AGGAGGAGCGGATTTCTCTC	12

Table 3: Banding pattern revealed by highly polymorphic SSR primers with extreme phenotypic scores as resistant and susceptible bulk

	Resistant			Mod. resistant		Highly susceptible				
Marker	Rathu Heenati	PTB33	ASD18	ADT 37	IR 38	CO 43	IR 50	BASMATI 370	TKM6	TN1
RM186	-	+	+	+	-	-	-	-	-	-
RM217	-	+	+	+	+	-	-	-	-	-
RM225	+	+	+	+	+	-	-	+	+	-
RM168	-	+	+	-	-	+	-	+	+	+
RM3331	+	+	+	-	+	+	-	-	-	-
RM 459	+	+	+	+	+	+	+	-	+	+
RM261	-	-	-	-	-	-	-	-	-	-

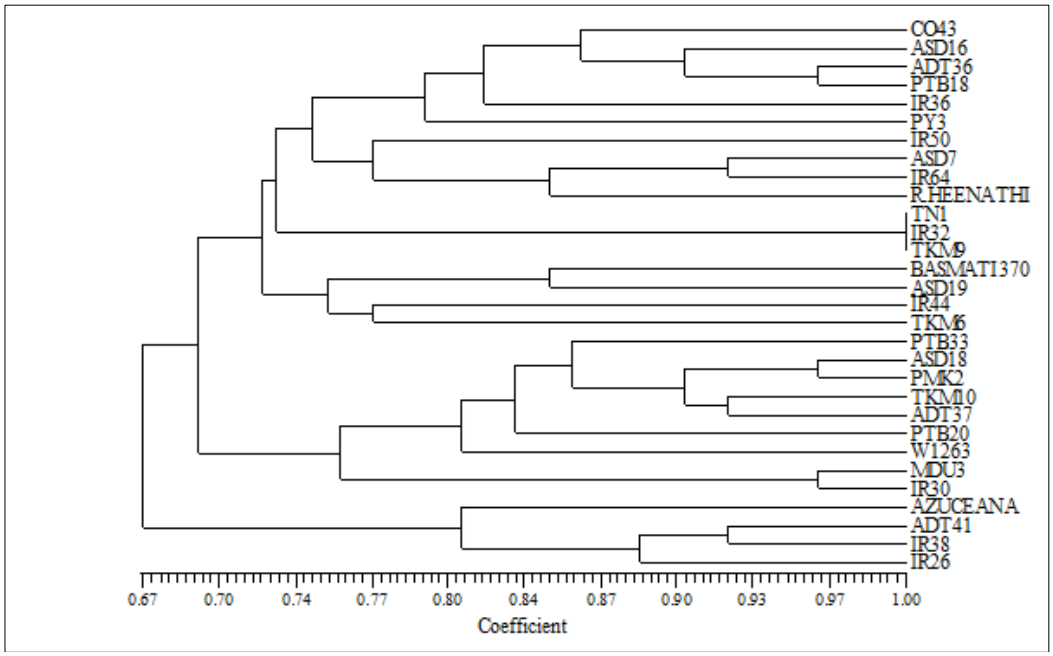

Fig 1: Dendrogram based on Dice similarity coefficient showing relationship among thirty rice varieties using SSR markers

Conclusion

In the present study, SSR markers were able to reveal greater genetic diversity among the rice varieties. SSR locus RM217 on chromosome # 6 was found to be putatively associated with BPH resistance. From this diversity analysis using DNA markers, selection of diverse resistant (PTB 33, Rathu Heenati and ASD 18) and susceptible parents (IR 50, TN 1, ADT 36, CO 43, Basmati 370) can be coupled for developing mapping populations to map and clone the gene/ QTLs for BPH since they have low similarity index values.

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