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MICROSATELLITE MARKER BASED GENETIC DIVERSITY ANALYSIS IN BROWN PLANT HOPPER RESISTANT RICE GENOTYPES

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Abstract

Genetic improvement mainly depends on extent of genetic variability present in the population. In the present study, a total of 38 SSR markers were used across 27 rice genotypes for their discrimination. Among these 21 markers showed polymorphism and number of alleles ranged from 2 to 5 with an average of 2.9 alleles per locus. The overall size of amplified fragments ranged from 95 to 270 bp. The polymorphic information content values varied from 0.14 to 0.72 with an average of 0.48 per locus. Cluster analysis was used to group cultivars by constructing dendrogram based on SSR markers analysis. All studied genotypes grouped into two main clusters at 23% similarity based on Jaccard's similarity index with additional sub clusters within each group. Based on this study, the larger range of similarity values for related cultivars using microsatellites provides greater confidence for the assessment of genetic diversity and relationships, which can be used in future breeding programs.

Introduction

Rice (*Oryza sativa* L.) as a major cereal crop, is one of the most diversified crop species due to its adaptation to a wide range of geographical, ecological and climatic regions. Rice is the most economically important food crop in many developing countries and has also become major crop in many developed countries, where its consumption has increased considerably, particularly in North America and the European Union (EU) due to food diversification and immigration. Rice provides about two-third of the caloric intake in Asia and one-third of caloric intake in Africa and Latin America (Shastry *et al.* 2000). India is remarkably rich in rice diversity, including cultivars, land races, wild and weedy relatives.

Brown plant hopper (BPH) is one of the most devastating pests of rice and can cause yield loss up to 60% (Panda and Khush 1995). The use of resistant varieties is an economical and effective way to control BPH. Consistent efforts have been made to identify genes for BPH resistance from various sources to develop resistant varieties. Genetic diversity is a pre-requisite for any crop improvement program as it helps in estimating and establishing of genetic relationship in germplasm collection, identifying diverse parental combinations to create segregating progenies with maximum genetic variability and superior recombinations for further selection and introgressing desirable genes from diverse germplasm (Islam *et al.* 2012).

There are several ways for estimation of diversity in germplasm, such as evaluation of phenotypic variation, biochemical and DNA polymorphisms. However, both phenotypic and biochemical characterizations are unreliable because they are environmentally challenged, labour demanding, numerically and phenologically limited. In contrast, DNA-based molecular markers are ubiquitous, repeatable, stable and highly reliable. Molecular markers are powerful tools in the assessment of genetic variation and in the elucidation of genetic relationships within and among

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species (Thomson *et al.* 2007). These markers have been utilized for many purposes, including genome mapping, gene tagging, estimation of genetic diversity, varietal differentiation and purity testing (Nagaraju *et al.* 2002).

Different molecular markers *viz.* RFLP, RAPD (Tingey and Deltufo 1993), simple sequence repeats (SSRs) (McCouch *et al.* 1997), AFLP and single nucleotide polymorphisms (SNPs) have been used to assess genetic diversity of various rice cultivars throughout the world (Joshi *et al.* 2000). Among different PCR based markers, the microsatellite markers based on simple sequence repeats (SSRs) are preferred over other molecular markers due to their easy application, high reproducibility, rapid analysis, low cost, easy scoring patterns and greater allelic diversity (Chen *et al.* 1997). These markers are distributed relatively uniformly throughout genome and detect a high level of allelic diversity in cultivated varieties and distantly related species (McCouch *et al.* 1997). A number of microsatellite markers have already been developed in rice and their primer sequences have been published (Wu and Tanksley 1993). Microsatellite markers have been effectively used to identify genetic variation among rice cultivars (Akagi *et al.* 1997). Information regarding genetic diversity at molecular level using DNA based molecular markers could be used to identify and characterize genetically unique germplasm that complement existing cultivars.

Hence, in the present investigation microsatellite markers were used for the genetic diversity analysis of a sample of 27 rice genotypes of which 14 were resistant and 13 were susceptible to brown plant hopper. This study was aimed at assessing the extent of genetic variation in the resistant and susceptible rice genotypes and to reveal genetic relationships among them for future use in selection, hybridization, and conservation of diverse gene pools.

Materials and Methods

Twenty seven improved rice genotypes were used for the molecular diversity analysis during 2011. The genotypes were screened in glass house condition for BPH resistance based on standard evaluation system (SES) (IRRI 1996) at the Department of Entomology, College of Agriculture, IGKV, Raipur (CG) during 2009 to 2011. Among rice genotypes used, 14 were resistant to BPH namely, Danwar2129, Danwar2511, Banshobira B 1021, Kariyabenikat K 1866, R1519-773-3-581-1, R1244-1246-1-605-1, R1723-1411-1-355-1 (all with score '0'), Uraibuta2408, R1243-1224-578-1, R1546-1321-3-178-1, R1723-1413-3-357-1, R1677-3473-1-4301-1, R1546-1328-1-90-1 and R1470-347-136-1-1(all with score '1'). Thirteen genotypes namely, Sampada, Karma Mahsuri, R1690-2163-2-213-1, R1685-2662-2-1903-1, R1614-914-1-400-1, R1711-2424-1-2514-1, R1576-1-1682-1-540-1, Samba Mahsuri, Mahamaya, Poornima, Danteshwari, Samleshwari and TN1 were susceptible to BPH.

Total genomic DNA was isolated from the leaves of three weeks old rice plants. Mini prep method was followed for the isolation of DNA. Purity and concentration of DNA was monitored spectrophotometrically at a wavelength of 260 and 280 nm using NanoDrop 2000c Spectrophotometer. All the DNA samples were diluted to a working concentration of 40 ng/µl for PCR reaction. Thirty eight primers were chosen randomly covering all the chromosomes or genomic regions for the genetic diversity analysis of 27 rice genotypes. Primers that showed polymorphic banding patterns were selected whereas primers with monomorphic banding patterns were excluded. Finally, 21 microsatellite primers were used for final polymerase chain reaction (PCR) amplification. Information regarding the original source, repeat motifs, primer sequences, expected length, chromosomal localizations and repeat types of the SSRs can be found in the Web database (http://www.gramene.org/db/markers.html). The amplification reaction conditions were adopted as described by Blair *et al.* (1999) with slight modifications. Prior to DNA amplification, 1.5 µl of diluted template DNA (40 ng/µl) of each genotype was dispensed in the bottom of PCR

plate. Separately PCR cocktail was prepared containing all required components in an Eppendorf tube and 18.5 µl of cocktail was added to each tube. PCR amplification reactions were done in 20 µl reaction mixtures, containing 1.5 µl of diluted template DNA, 0.5 µl of each forward and reverse primer, 1 µl of 1 mM dNTPs, 2 µl of 10x PCR buffer, 0.5 µl of *Taq* DNA polymerase, 0.5 µl of 25 mM $MgCl_2$ and 13.5 µl of ddH_2O. A DNA thermal cycler (Veriti 96 well thermal cycler, Applied Biosystems) was used along with the following PCR profile: An initial denaturation step for 5 min at 94°C (hot start and strand separation), followed by 35 cycles of denaturation (94°C), annealing (55°C) and primer elongation (72°C) for 1 min each and then a final extension at 72°C for 7 min. Amplified products were stored at –20°C until further use.

Prior to electrophoresis, each PCR product was mixed with 3 µl of 6X gel loading dye (bromophenol blue and sucrose) and immediately electrophoresis was carried out in a mini vertical electrophoresis tank run on 5% polyacrylamide gel in 1X TBE buffer. Ten micro liters of the sample was loaded with a 50 bp DNA step up ladder as molecular marker in the wells of the gel and run at a constant 180 volts for 45 minutes. The DNA bands were visualized by staining gel 1% ethidium bromide solution and photographed under UV light using a GelDocXR (BioRad) system.

All of the genotypes were scored for the presence (score '1') and absence (score '0') of the SSR bands. Ethidium bromide staining of gels generally showed several bands. The size of the most intensively amplified band for each microsatellite marker was determined based on its electrophoretic mobility relative to molecular weight markers (increments of 50). Clearly resolved unambiguous bands were scored visually for their presence or absence with each primer. 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. The score obtained using all the primers in the SSR analysis were pooled to create a binary data matrix and used to construct a dendrogram using UPGMA (Unweighted Pair Group Method of Arithmetic Means) algorithm. The pair wise similarity between isolates and polymorphic bands was calculated using Jaccord's coefficient. Polymorphic information content (PIC) values were calculated for each of the SSR loci according to the method of Anderson *et al.* (1993):

$$PIC_i = 1 - \sum_{j=1}^{n} Pij^2$$

where, Pij is the frequency of the j^{th} allele for the i^{th} marker, and is summed over n alleles. The calculation was based on the number of alleles/locus. Genetic similarities were estimated from the matrix of binary data using Jaccard's coefficient. The similarity coefficients were used for cluster analysis of the rice cultivars utilizing the UPGMA. The analysis and dendrogram construction were performed using the NTSYS-pc version 2.02 (Rohlf 1999).

Results and Discussion

Thirty eight microsatellite markers covering all 12 chromosomes were utilized to assess genetic diversity among 27 rice genotypes. Among 38 SSR markers, 21 spread on chromosomes 1, 2, 3, 5, 6, 7, 8, 9, 10 and 12 were identified as polymorphic. Table 1 show that, a total number of 61 alleles were detected at the loci of the 21 SSR markers across the 27 rice genotypes. The number of alleles per locus generated by each marker varied from 2 to 5 alleles with an average of 2.9 alleles per locus. The highest number of alleles (5.0) was detected in the locus RM152, RM341 and RM228 while the lowest number of alleles (2.0) was detected on each of locus RM475, RM464, RM498, RM477, RM468, RM247, RM231, RM220 and RM411. The overall size of the amplified product varied from 95 bp (approx.) (RM490) to 270 bp (approx.) (RM468).

The size difference between the smallest and the largest band at a given SSR locus varied from 5 bp (RM411) to 40 bp (RM505, RM488 and RM228).

Table 1. Banding pattern for microsatellite markers identified in 27 genotypes of rice.

Sl. No.	SSR markers	Chromosome No.	No. of bands	SSR motifs	Range of band size (bp)	Difference	PIC
1.	RM5	1	3	(GA)14	110-130	20	0.61
2.	RM152	8	5	(GGC)10	145-165	20	0.70
3.	RM190	6	3	(CT)11	115-135	20	0.51
4.	RM220	1	2	(CT)17	115-125	10	0.39
5.	RM228	10	5	(CA)6(GA)36	110-150	40	0.72
6.	RM231	3	2	(CT)16	170-190	20	0.51
7.	RM247	12	2	(CT)16	130-160	30	0.50
8.	RM288	9	3	(GA)7G6(GA)7	130-160	30	0.66
9.	RM341	2	5	(CTT)20	150-180	30	0.49
10.	RM411	3	2	(GTT)7	110-115	05	0.49
11.	RM421	5	3	(AGAT)6	240-250	10	0.64
12.	RM449	1	3	(AG)12	110-125	15	0.55
13.	RM464	9	2	(AT)21	230-260	30	0.42
14.	RM468	3	2	(TAT)8	260-270	10	0.48
15.	RM475	2	2	(TATC)8	190-200	10	0.48
16.	RM477	8	2	(AATT)5	220-225	05	0.14
17.	RM488	1	3	(GA)17	170-210	40	0.54
18.	RM490	1	4	(CT)13	95-120	25	0.56
19.	RM498	2	2	(CA)10	215-225	10	0.26
20.	RM505	7	3	(CT)12	170-210	40	0.22
21.	RM544	8	3	(TC)9	230-250	20	0.38
Average							0.48

Wong *et al.* (2009) reported the genetic relationship and diversity analysis among 8 Bario rice cultivars using 12 SSR primers, detecting a total of 31 alleles and average number of alleles per locus was 2.6, which is lower than our report. Similar results for low number of alleles per locus were obtained by Vanniarajan *et al.* (2012) (2.5 alleles per locus). In contrast, the average number of alleles detected in the present study was lower than the average number of alleles reported by Jain *et al.* (2004), Lu *et al.* (2005), Jayamani *et al.* (2007), Thomson *et al.* (2007), Pervaiz *et al.* (2010) and Upadhyay *et al.* (2011), who reported an average of 6.8, 7.8, 6.57, 7.7, 13, 4.4 and 4.35 alleles per locus using rice subspecies, Indian quality rice germplasm, US rice cultivars, a diverse collection of Portuguese rice, Indonesian rice germplasm (both indica and japonica), Pakistani rice landraces and Indian rice varieties, respectively. There is such big variability using SSRs in the number of alleles detected per locus and this inconsistency might be due to the diverse genotypes used and selection of SSR primers with scorable alleles.

The polymorphism among the rice genotypes is shown in primer RM228 (Fig. 1). RM152, RM341 and RM228 detected 5 alleles followed by RM490 (4 alleles) and RM5, RM190, RM288, RM421, RM449, RM488, RM505 and RM544 (3 alleles). This suggests that these markers could be potentially used for molecular diversity of rice genotypes from various sources. However, there

were a number of markers which produced only few alleles. Nine markers produced two alleles and despite their ability to produce only few alleles, they were robust enough to distinguish specifically diverse genotypes or different accessions of the same genotype.

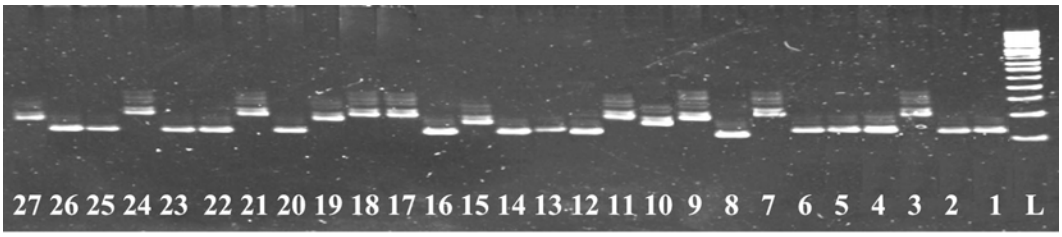

Fig. 1. DNA profiles of 27 rice genotypes SSR primer RM228.

Lane: L - DNA Ladder, 1. R1546-1321-3-178-1, 2. R1723-1413-3-357-1, 3. R1677-3473-1-4301-1, 4. Danwar 2511, 5. Banshobira B 1021, 6. Kariyabenikat K 1866, 7. R1243-1224-578-1, 8. R1723-1411-1-355-1, 9. R1546-1328-1-90-1, 10. R1519-773-3-581-1, 11. R1244-1246-1-605-1, 12. R1470-347-136-1-1, 13. Danwar 2129, 14. Uraibuta 2408, 15. Sampada, 16. Karma Mahsuri, 17. R 1690-2163-2-213-1, 18. R1685-2662-2-1903-1, 19. R1614-914-1-400-1, 20.R1711-2424-1-2514-1, 21. R1576-1-1682-1-540-1, 22. Samba Mahsuri, 23. Mahamaya, 24. Poornima, 25. Danteshwari, 26. Samleshwari and 27. TN1. (Resistant genotypes: Lane No. 1 to 14; Susceptible genotypes: Lane No. 15 to 27)

PIC value refers to the value of a marker for detecting polymorphism within a population, depending on the number of detectable alleles and the distribution of their frequency, thus it provides an estimate of the discriminating power of the marker (Nagy *et al.* 2012). The PIC value of each marker, which can be evaluated on the basis of its alleles, varied greatly for all tested SSR loci from 0.14 (RM477) to 0.72 (RM228) with an average of 0.48 per locus (Table 3). The low PIC values were observed for ten SSR markers. Lower PIC value may be the result of closely related genotypes. Low PIC values for some other primers were earlier reported by Juneja *et al.* (2006). The highest PIC value 0.72 was obtained for RM228 followed respectively by RM152 (0.70), RM288 (0.66), RM421 (0.64), RM5 (0.61), RM490 (0.56), RM449 (0.55), RM488 (0.54), RM231 (0.51) and RM190 (0.51). It indicated that these SSR markers used in this were highly informative because only PIC values higher than 0.5 indicate high polymorphism.

In the present study, the level of polymorphism determined by the PIC value (mean = 0.48) is consistent with previous estimates of microsatellite marker analysis in rice by Lu *et al.* 2005 (0.028 to 0.881, mean = 0.463), Prabakaran *et al.* 2010 (0.28 to 0.57, mean = 0.43) and Sajib *et al.* 2012 (0.14 to 0.71, mean = 0.48). The PIC value was lower than that of previously reported by Jain *et al.* 2004 (0.24 to 0.92, mean = 0. 61), Jayamani *et al.* 2007 (0.179 to 0.894, mean = 0.66), Thomson *et al.* 2007 (0.34 to 0.88, mean = 0. 66) and Borba *et al.* 2009 (0.19 to 0.90, mean = 0. 75). Upadhyay *et al.* (2011) also reported the average PIC value of 0.78 among popular rice varieties of India. The result revealed that markers RM228 would be best in analyzing 27 rice genotypes followed by RM152, RM288, RM421, RM5 and so on. Thus, the PIC value indicates that all these primers were highly informative and capable of distinguishing between genotypes.

The genetic relationships among rice genotypes are presented in a dendrogram based on informative microsatellite alleles (Fig. 2). All genotypes clearly grouped into two major clusters in the dendrogram at 23% similarity based on Jaccard's similarity index. The similarity coefficients had ranged from 0.23 to 0.91, but mostly concentrated from 0.36 to 0.85. Cluster I was the big group comprising of 23 genotypes and cluster II four genotypes. Cluster I has further divided into two cluster i.e. cluster I A (four genotypes) and cluster I B (19 genotypes) at the genetic similarity coefficient of 0.31. Cluster II has a few genotypes which is further divided into two cluster *viz.*,

cluster II A (three genotypes) and cluster II B (one genotype). All the genotypes of cluster II were highly resistant to Brown plant hopper. Analysis of the clusters of dendrogram revealed that genotypes Danwar2511 and Kariyabenikat K 1866 in cluster II showed highest genetic similarity of 91 per cent which were highly resistant to brown plant hopper. Also the genotypes Danteshwari and Samleshwari in cluster I showed highest genetic similarity of 91 per cent which are tolerant to drought, early maturing and popular varieties of the state. However, both of them exhibited the susceptible reaction to brown plant hopper. Similarly in cluster I breeding lines R1519-773-3-58-1 and R1244-1246-1-605-1 showed 76 per cent similarities which are highly resistant to brown plant hopper. Thus cluster analysis based on SSR markers could not separate rice genotypes into

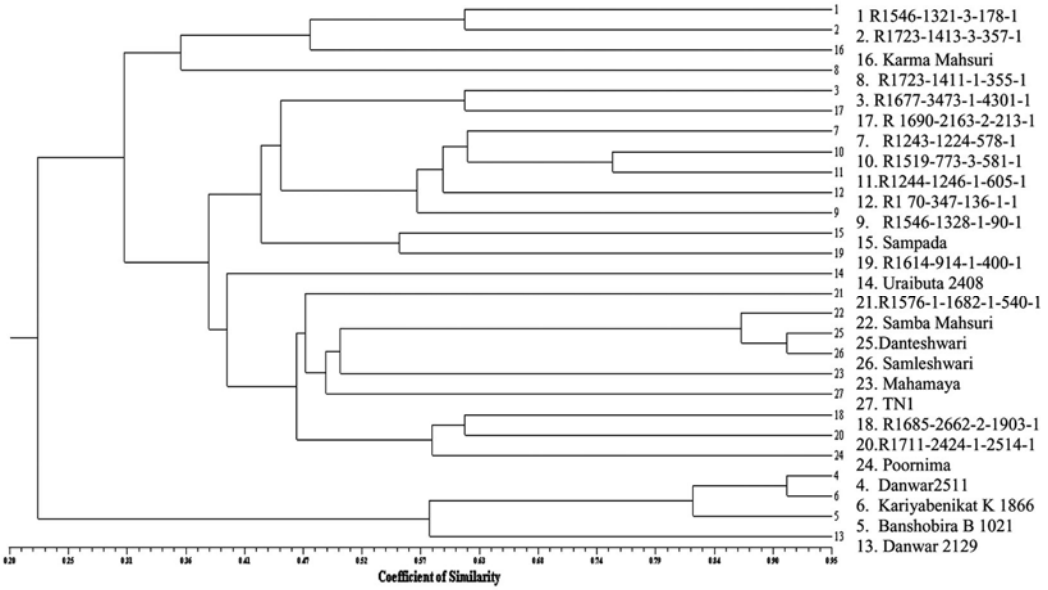

Fig. 2. Dendrogram derived from microsatellite (SSR) analysis of 27 rice genotypes.

groups according to the brown plant hopper resistance although it could classify rice genotypes by the location and association with genetic origin. In this study, the larger range of similarity values for cultivars revealed by microsatellite markers provides greater confidence for the assessments of genetic diversity and relationships, which can be used in future breeding programs. The evaluation of genetic similarity and cluster analysis together with brown plant hopper resistance provides some useful guides for assisting plant breeders in selecting genetically diverse parents for crossing programme and also assist in broadening germplasm-based rice breeding programs in the near future. Thanh *et al.* (1999) found that the SSR-based dendrogram resolved the 31 Vietnamese upland rice accessions into two major groups. The rice genotypes were classified and grouped into 11 distinct groups (Chakravarthi and Naravaneni 2006). Yu *et al.* (2004) studied cluster analysis of the 223 accessions parental lines of rice and showed three major groups and nine sub groups. The dendrogram revealed that the genotypes that are derivatives of genetically similar type clustered together. El-Malky *et al.* (2007) reported the ability of SSR makers to divide the varieties into two groups, one included the indica varieties and the other included the japonica varieties.

The present work revealed genetic variation and relatedness among the 27 rice genotypes. The threat of BPH to rice has resulted in search of genes in the available germplasm showing resistance. To exploit maximum heterosis as well as to develop desired brown plant hopper resistant variety hybridization programme may be initiated between the genotypes in the two clusters for getting transgressive segregants since these genotypes showed maximum diversity. In addition, it will help in identifying efficient strategies for the sustainable management of the genetic resources of rice crops.

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