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GENETIC DIVERSITY FOR SUBMERGENCE TOLERANCE IN RICE (*Oryza sativa* L.) UTILIZING SSR MARKERS

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ABSTRACT

The present study was aimed to analyze molecular diversity of submergence tolerant genotypes of rice. Thirty four SSR primers were used to generate allelic variants (4 alleles in RM 23843 and 27 in RM 23662). Altogether 410 allelic variants were detected at 42 SSR loci with a mean of 9.76 alleles per locus. Analysis of divergence pattern based on SSR markers allowed differentiation and classification of rice varieties into 8 clusters. A large range of similarity coefficient revealed by SSR markers provided greater confidence for the assessment of genetic divergence and interrelationship among the submergence tolerant rice genotypes. A perusal of similarity coefficients reflected a very high degree of similarity between rice genotypes 'Anh Hsung Seln' (C 11) and 'Hsung Teing', whereas 'FR 13A' and 'Swarna Sub-1' were more diverse for breeding programme to generate more recombinants. Use of SSR markers appeared more efficient in achieving unique and unambiguous characterization and differentiation of varieties used in the present study. The SSR analysis also revealed unique or variety-specific allele which could be useful as DNA fingerprints in the identification and preservation of different genotype and to develop highly submergence tolerance varieties.

Key words: Allele, divergence, loci, rice, submergence

INTRODUCTION

Rice (*Oryza sativa* L.) belongs to the family Poaceae and is staple food of half of the world's population. The present population of world is 7.5 billion with current growth rate 1.11% per year as per UNDP approximations (Rao *et al.*, 2016). The latest United Nations projections indicate that world population will reach 10 billion in the year 2056 (UNPD, 2015). This emphasizes that rice production must increase by 40-50% to meet the growing demand (Ray *et al*., 2018). About 32.4% of India's total rice area i.e. 15 million ha lies under rainfed lowlands. Rainfed lowlands constitute highly fragile ecosystems and are always prone to flash-floods (submergence). Among rice-growing ecosystems, the rainfed lowland areas are most challenging one owing to the prevalence of many abiotic and biotic stresses (Iftekharuddaula, 2016). Among the 42 biotic and abiotic stresses affecting rice production, submergence has been identified as the 3rd most important constraint for higher rice productivity (Sarkar and Bhattacharjee, 2011). About 4 million tonnes of rice is annually lost due to the flooding (IRRI, 2008). Rice productivity in areas planted in this way is low and unstable, averaging <2.0 t ha^{-1} in rainfed lowlands and <1.5 t ha^{-1} in flood-prone areas as compared to >5.0 t ha^{-1} in input-intensive irrigated systems (Ismail *et al.*, 2013; Singh *et al.*, 2013). For any

breeding programme, the characterization of germplasm is pre-requisite. Jha (2012) suggested the importance of biochemical markers to distinguish wide and intraspecific variations. Molecular markers developed for differentiation of genotypes and assessment of genetic diversity are reliable and remain unaffected across different growth stages, seasons, locations and agronomic practices (Joshi and Behera, 2006; Rathi and Sharma, 2012). They can be easily amplified by polymerase chain reaction by using the primers specific to unique flanking sequences of SSR; and the polymorphic amplified fragments can be produced as a result of difference in the number of repeat units (Zeng, 2004; Rathi and Sharma, 2012). In view of above, the present study was aimed to assess genetic variation and grouping of genotypes at the molecular level for rice improvement.

MATERIALS AND METHODS

Plant material and design

Rice genotypes were procured from the Department of Plant Breeding & Genetics, RPCAU, Pusa (India). Plants were raised in Research Farm, Borlaugh Institute of South Asia, Pusa (India) in a randomized complete block design in quadruplicates. Each genotype was transplanted in 4 rows of 4 m long. The plant spacing was maintained at 45 cm x 25 cm. Phenotyping was done in the year 2016.

DNA extraction and quantification

Genomic DNA was extracted from the tips of young leaves of rice plant by CTAB method (Doyle and Doyle, 1987). The absorbance of DNA samples was measured by UV spectrophotometer (Varian-make) at 260 nm so as to quantify DNA purity.

Thirty-four SSR primers used (Table 1) were obtained from Eurofins MWG Operon Inc., Louisville, Kentucky, USA. The primers in vials were centrifuged at 4000 rpm for 10 min before and after the addition of 1X TE buffer. TE was added to each vial to obtain the desired concentration of primer stock solution (100 µM). The primer stock solutions were diluted appropriately to 10 µM and stored at -20°C. The most important components of reaction, like concentration of primer and template DNA, which were likely to affect the PCR process were arranged in an orthogonal array (primer concentration - 0.2, 0.5, 1.0, 1.0 and 2.0 µM and DNA concentration - 2, 3, 4 and 5 ng μL^{-1}). The other components of PCR process were kept constant. The amplified products were separated electrophoretically on 0.8% agarose gel in 0.5 x TBE buffer, visualized and photographed under UV light in a gel documentation hood (Bio-Rad XR plus) after staining with ethidium bromide (EtBr).

Scoring and analysis of bands

Clear visible bands were coded in a binary form by denoting '0' and '1' indicated the absence and presence of bands, respectively, in each genotype. The data were used for further calculations. To measure the informativeness of SSR markers, the polymorphism information content (PIC) for each marker was calculated according to the formula given by (Anderson *et al.*, 1993).

$$PIC_i = 1 - \sum_{j=1}^{k} P^2_{ij}$$

Where, k is the total number of alleles detected for a locus of a marker and Pi is the frequency of the i^{th} allele in the set of 19 varieties investigated.

The PIC value for each marker was used to justify polymorphic information, and the mean PIC value for a group of individuals implied the genetic diversity within a group. Both the PIC for each marker and mean PIC for each group were determined. To describe the genetic relationship, microsatellite data were used to estimate the genetic distance, based on Jaccard similarity coefficient, by drawing the phenon line at 15 similarity units in dendrogram. The entries (19) were divided into 8 clusters for the purpose of deriving inference on divergence pattern amongst the

entries at molecular level. The nature and extent of diversity between submergence-tolerance related rice varieties were assessed by identifying the clusters at appropriate phenon levels. The cluster analysis was done by using NTSYS-pc version 2.1m.

RESULTS AND DISCUSSION

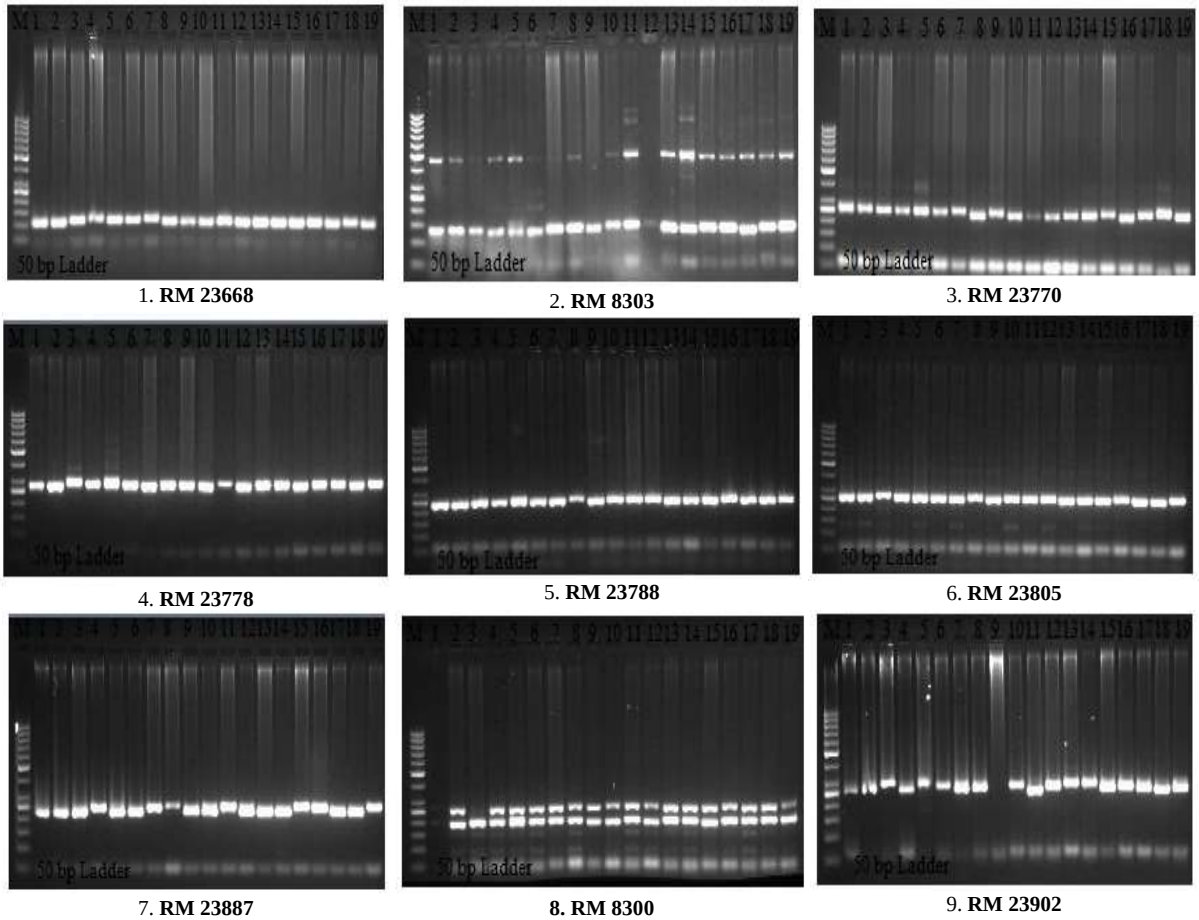
Microsatellite (SSR) markers are useful for different applications in rice breeding and SSR markers even in very less number can give a better genetic diversity spectrum due to their multi-allelic and highly polymorphic nature (Singh *et al.* 2016). Reports suggest that genetic diversity in crop varieties released over the years fluctuates in successive time periods (Upadhyay *et al.* 2012). Over the last few centuries, rice has faced diversity loss (Choudhary *et al.* 2013). The present study exhibited the utility of SSR markers in revealing the extent of genetic diversity at molecular level among 19 submergence tolerant rice genotypes. A total of 34 SSR primer pairs were used in screening

Table 1: Analysis of primer pairs used for the amplification of genomic DNA extracted from nineteen rice varieties

Primers	No. of loci	Product size (bp)	No. of alleles	No. of unique alleles	Percentage of unique alleles	PIC	No. of entries having null alleles
RM 23668	1	102-128	11	6	54.54	0.872	0
RM 23679	1	211-253	13	8	61.53	0.908	0
RM 8303	1	129-158	12	6	50.00	0.903	0
RM 23770	1	210-361	14	11	78.57	0.903	0
RM 23778	1	274-299	6	1	16.66	0.759	0
RM 23788	1	234-283	12	7	58.33	0.903	0
RM 23805	1	225-263	9	3	33.33	0.864	0
RM 23831	1	159-194	7	2	28.57	0.814	0
RM 23843	1	284-303	4	2	50.00	0.693	11
RM 23887	1	225-260	10	5	50.00	0.858	0
RM 8300	2	191-291	19	10	52.63	0.925	0
RM 23901	2	118-299	15	13	86.66	0.927	0
RM 23902	1	268-312	12	7	58.33	0.913	1
RM 23915	1	191-251	14	10	71.42	0.914	0
RM 23917	1	230-247	6	1	16.66	0.792	0
RM 23922	1	173-248	9	3	33.33	0.880	4
RM 23928	1	153-277	14	11	78.57	0.919	0
RM 23958	2	164-270	26	20	76.92	0.949	0
RM23996	1	253-305	11	5	45.45	0.891	0
RM 24005	1	217-269	14	10	71.42	0.914	0
RM 24103	1	105-205	12	9	75.00	0.898	3
RM 105	1	103-131	9	3	33.33	0.864	0
RM 215	3	152-542	20	8	40.00	0.922	0
RM 257	2	111-228	22	10	45.45	0.941	0
RM 316	2	166-220	11	5	45.45	0.871	0
RM 23662	2	148-315	27	20	74.07	0.956	0
RM 24013	1	153-203	8	5	62.50	0.781	0
RM 910	1	126-191	13	11	84.61	0.858	0
RM 7175	1	90-111	9	5	55.55	0.836	0
ART 5	1	220-265	10	5	50.00	0.897	0
SC 3	1	197-209	5	0	0	0.786	0
RM 296	1	112-129	7	2	28.57	0.819	0
Sub $1BC_2$	1	254-337	11	6	54.54	0.869	0
RM 23865	1	140-158	8	3	37.50	0.814	1

study, which was earlier identified in the genomic regions of chromosome 9 of the rice genome (Table 1). A total of 42 loci were assigned to the 34 SSR primer pairs. Analysis of bands produced by primer pair-based amplification of genomic DNA extracted from the 19 varieties of rice in each lane (Fig. 1). A total of 410 allelic variants were detected with an average of 9.76 alleles per locus. The number of alleles per locus ranged from 4 in 'RM 23843' to 27 in 'RM 23662'. This revealed significant differences in allelic diversity among various microsatellite loci. Many studies have reported remarkable differences in allelic diversity among various microsatellite loci (Iftekharuddaula, 2015).

The alleles revealed by markers showed a higher degree of polymorphism. The allelic polymorphic information content varied from 0.693 in case of 'RM 23843' to 0.956 in case of 'RM 23662' with an average of 0.871 per primer. Null alleles are known to arise as a consequence of sequence changes at the primer binding sites. The occurrence of null alleles was also noticed in various genotypes for a particular locus, whenever an amplification product could not be detected in a specific SSR primer pair combination. Null alleles have also been detected by Kalinowaski and Taper (2006). Presence of stutter bands was detected in the present study. Stutter bands indicated the presence of minor products amplified in PCR that had a lower intensity than the main allele and normally lacked or had extra units. Such bands were observed in case of tri-nucleotide SSR sequence detected by primer pairs 'RM 8303', 'RM 23770,' 'RM 23805', 'RM 23831', 'RM 8300', 'RM 23901', 'RM 23915', 'RM 23917', 'RM 23922', 'RM 23928', 'RM 23958', 'RM 23996', 'RM 24103', 'RM 105', 'RM 215', 'RM 316', 'RM 23662', 'RM 24013', 'RM 910', 'RM 7175', 'ART 5', 'SC3', 'Sub 1 BC2' and 'RM 23865'. The SSR loci with di-nucleotide repeat motifs, in general, tended to detect greater number of alleles as revealed by 'RM 23958', 'RM 257', 'RM



1. **RM 23668** 2. **RM 8303** 3. **RM 23770**

4. **RM 23778** 5. **RM 23788** 6. **RM 23805**

7. **RM 23887** **8. RM 8300** 9. **RM 23902**

Fig. 1: The loci which were assigned to the thirty four SSR primer pairs

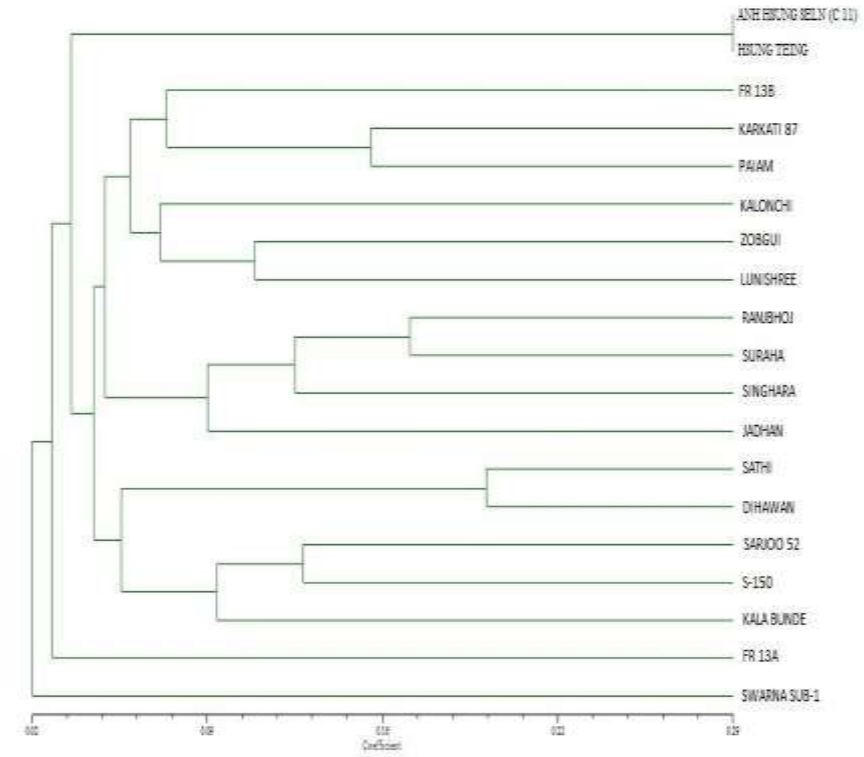


Fig. 2: Dendrogram based on average Jaccard's similarity coefficient for 34 SSR primer pairs among 19 rice genotypes

215', 'RM 23901', 'RM 23915', 'RM 316', 'RM 23805', 'RM 23865', 'RM 24103', 'RM 24005', 'RM 23996', 'RM 23928', 'RM 23922', 'RM 23917' and 'RM 23843' than the 'STR' loci with tri-nucleotide repeat motifs as revealed by 'RM 23668', 'RM 23770', 'RM 23902', 'RM 105', 'RM 24013', 'RM 910', 'ART 5', 'SC3', 'RM 296' and 'Sub 1BC2' or tetra-nucleotide repeat motifs as revealed by 'RM 23679', 'RM 8303', 'RM 23778', 'RM 23788', 'RM 23831', 'RM 23887', 'RM 8300', 'RM 23662' and 'RM 7175'. Stutter bands were probably produced by the slippage of polymerase amplification and the factors that influenced the proportion of stutter band to the main allele were the repeat number, number of PCR cycles, length and the characteristics of repeat sequence. Since a change in the number of repeats leads to the generation of allelic variants because of variation in the size of SSR allele, the total repeat count of tri-nucleotide SSR loci was found associated with the number of alleles. Larger the repeat number involved in SSR locus, the larger was the number of identified alleles. It can be observed an increase in a number of allele with repeat number of microsatellites used and their relative distance from centromere, which was not dependent on the motif of microsatellites (Prathyusha *et al.*, 2009, Biswas *et al.*, 2012). Allelic diversity data was used to produce a dendrogram in order to elucidate relationship between 19 rice genotypes. The similarity coefficient was estimated on the basis of Jaccard's coefficient (Rohlf, 2000). The estimates of similarity coefficients, ranging from 0 to 1, indicated considerably a greater extent of variation among the rice varieties under evaluation. The large range of similarity coefficient revealed by SSR markers provided greater confidence for the assessment of genetic divergence and interrelationship among the submergence tolerant rice genotypes. A perusal of similarity coefficients clearly reflected a very high degree of similarity between rice genotypes 'Anh Hsung Seln (C 11)' and 'Hsung Teing', whereas 'FR 13A' and 'Swarna Sub-1' were found more diverse, so may be used in breeding programme to generate more recombinants. Further, the phenon line divided all other sub-clusters into mono-genotypic clusters (Fig. 2). The pattern of clustering and sub-clustering showed that the genotypes with distinct DNA profiles likely to contain novel genes and are likely to be unique and agronomically useful, so may be exploited for crop improvement.

The present study demonstrated that the use of molecular markers is important for submergence prone areas (Ray *et al.*, 2014). Ultimately, transfer sub-1 QTL from 'Swarna sub-1' donor to locally adapted 'Rajendra Mansuri' recipient variety through marker-assisted back-crossing using SSR marker like 'Sub-1 BC2', 'RM 8300' and 'RM 23865' later are flanking

markers and it will be used to introgress sub-1 segment to high yielding varieties for development of highly submergence tolerant rice.

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