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MOLECULAR DIVERSITY ANALYSIS FOR SUBMERGENCE TOLERANCE IN RICE (ORYZA SATIVA L.) USING SSR MARKERS

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ABSTRACT

A study was conducted to analyze the molecular diversity of nineteen submergence tolerant genotypes during *Kharif*– 2016. A panel of thirty four SSR primers generated allelic variants ranging from four in the cases of RM 23843 to twenty seven in the cases of RM 23662. Altogether 410 allelic variants were detected at 42 SSR loci with an average of 9.76 alleles per locus. Analysis of divergence pattern based on SSR markers allowed differentiation and classification of rice varieties into eight clusters. 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 that a very high degree of similarity exists between rice genotypes Anh Hsung Seln (C 11) and Hsung Teing whereas FR 13A and Swarna Sub-1 found more diverse, may be used in 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 rice varieties.

KEY WORDS
Rice
divergence
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INTRODUCTION

Rice, Oryza sativa L. (2n = 24) belonging to the family Poaceae and subfamily Oryzoideae is the staple food for half of the world's population and occupies almost one-fifth of the total land area covered under cereals. About 32.4% of India's total rice area, *i.e.*, 15 M ha is under rainfed lowlands. Rainfed lowlands constitute highly fragile ecosystems, always prone to flash-floods (submergence). Among the 42 biotic and abiotic stresses affecting rice production, submergence has been identified as the third most important constraint for higher rice productivity (Sarkar and Bhattacharjee ,2011). Scientists have estimated that 4 million tons of rice is being lost every year because of flooding (IRRI, 2008). According to an estimate of National Bureau of Soil Survey and Land Use Planning nearly 3.3 M ha of land is affected by flood of varying degree. For any breeding programme, characterization of the germplasm is essential to formulate the group for their effective utilization. Jha *et al*. (2012) suggested the importance of bio-chemical marker (SDS PAGE profiling) to distinguish the wide and intra specific variation. Molecular markers developed for the differentiation of genotypes and assessment of genetic diversity are reliable and remain unaffected across different growth stages, seasons, location and agronomic practices (Joshi and Behera, 2006; Rathi and Sharma, 2012). Microsatellites or Simple Sequence Repeats (SSR) are tandemly repeated sequence motifs ubiquitously distributed throughout the rice genome. They can be easily amplified by polymerase chain reaction using primers specific to the unique flanking sequences of the SSR and polymorphic amplified fragments can be produced due to difference in the number of the repeat units (Zeng *et al*., 2004; Rathi and Sharma, 2012). Keeping the above facts under consideration, the present investigation was done to assess the genetic variation and grouping of genotypes at molecular level for rice improvement.

MATERIALS AND METHODS

Plant material and experimentation

Nineteen rice genotypes were received from Department of Plant Breeding & Genetics, RAU, Pusa; developed by different centers are presented in Table 1. Plants were raised in research field of Dr.RPCAU, Pusa in randomized complete block design (RCBD) in quadruplicates. Each genotype was grown in a four rows of 4 m length. The spacing was maintained at 45 x 25 cm inter-row and inter-plant geometry, respectively. All recommended agronomic practices were followed during the crop period.

DNA extraction and quantification

Genomic DNA was isolated from young leaves by CTAB method described by Doyle and Doyle (1987). Purification of the isolated DNA was done to remove RNA, proteins and polysaccharides, which are considered to be the major contaminants in the DNA precipitate. Inclusion of CTAB in the DNA extraction buffer helps elimination of polysaccharides. RNA was removed by RNAase treatment and proteins were removed by phenol-chloroform extraction. The absorbance (optical density) of the purified DNA sample obtained after the purification step was recorded by UV Varian-Make Spectrophotometer at 260 nm in a quartz cuvette in order to quantify the purity of DNA. Microsatellite primers, polymerase chain reaction (PCR) and electrophoresis.

Thirty four SSR primers were obtained from Eurofinsmwg/ operon (Table 2). The primer vials were centrifuged before and after the addition of 1X TE buffer to the vials. Mentioned volume of the TE was added to each vial so as to obtain the desired concentration of the primer stock solution (100 μM). The primer stock solutions were diluted appropriately to 10 μM for further use. Diluted primers were stored at -20°C. Standardization of genomic DNA has been attempted to optimize the PCR reaction for amplification. A method designed to reveal the effects and interactions of specific reaction components simultaneously using a few reaction was adopted. In this method, the most important components of reaction such as concentration of primer and template DNA which were likely to affect the PCR process, were arranged in orthogonal array (Primer concentration- 0.2, 0.5, 1.0, 1.0, 2.0 μM and DNA concentration 2.3, 4, 5 ng/μl). The other components of PCR process were kept constant. The amplified products were separated electrophoretically on 0.8 per cent agarose gel in 0.5 x TBE buffer, visualized and photographed over a UV light in gel doc 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 the SSR markers, the polymorphism information content (PIC) for each marker was calculated according to the formula given by Anderson et al. (1993).

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

Where,

k is the total number of alleles detected for a locus of a marker and

Pi the frequency of the ith allele in the set of 19 varieties investigated.

The PIC value for each marker was used to justify the polymorphic information, and the mean PIC value for a group of individuals implies the genetic diversity within the 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 and 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 characterization due to their high level of polymorphism and easy handling (Chen *et al*., 1997) and are used to evaluate genetic diversity. Polymorphism in SSR is generally believed to be the result of replication error (Moxon and Wills, 1999), which occurs at a rate higher than the mutation in a non-repetitive DNA (Wierdl *et al*., 1997). The present study addressed the utility of SSR markers in revealing the extent of genetic diversity at the molecular level among 19 submergence tolerant rice genotypes. A total of 34 SSR primer pairs were used for the purpose of screening, which were earlier identified in the genomic regions of chromosome 9 of the ricegenome; are presented in Table 2. A total of 42 loci were assigned to the thirty four SSR primer pairs (Fig 1-18). A total of four hundred and ten allelic variants were detected among them with an average of 9.76 alleles per locus (Table 3). The number of alleles per locus ranged from four in the cases of RM 23843 to twenty seven in thecases of RM 23662. This revealed significant differences in allelic diversity among various microsatellite loci. Many studies have also reported remarkable differences in allelic diversity among various microsatellite loci (McCouch *et al*., 2001; Ravi *et al*., 2003; Ram *et al*., 2007; Herrera *et al*., 2008). The alleles revealed by markers showed a higher degree of polymorphism.

The allelic polymorphic information content (PIC) varied from 0.693 in the case of RM 23843 to 0.956 in the case of RM 23662 with an average of 0.871 per primer. (Table 3). Null alleles are known to arise as a consequence of sequence changes at the primer binding site(s). 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 were also detected by Kalinowaski, S.T. and Taper, M.L. (2006). Presence

Table 1: List of submergence tolerant genotypes and their source used in the present investigation

Sl. No.	Genotypes	Source
M	50 bp Ladder	Thermo Fisher Scientific
1	ANH HSUNG SELECTION (CI 1)	IRRI, Philippines
2	HSUNG TIENG	IRRI, Philippines
3	FR 13A	CRRI, Cuttack, India
4	FR 13B	CRRI, Cuttack, India
5	KARKATI 87	IRRI, Philippines
6	PAIAM	IRRI, Philippines
7	KALONCHI	IRRI, Philippines
8	ZOBGUI	IRRI, Philippines
9	LUNISHREE	CRRI, Cuttack, India
10	RAJBHOG	IRRI, Philippines
11	SINGHARA	IRRI, Philippines
12	SURAHA	NBPGR, New Delhi, India
13	SATHI	NBPGR, New Delhi, India
14	DIHAWAN	NBPGR, New Delhi, India
15	JADHAN	NBPGR, New Delhi, India
16	SARJOO 52	NBPGR, New Delhi, India
17	S-150	NBPGR, New Delhi, India
18	KALA BUNDE	CRRI, Cuttack, India
19	SWARNA SUB-1	GENETICS Seeds

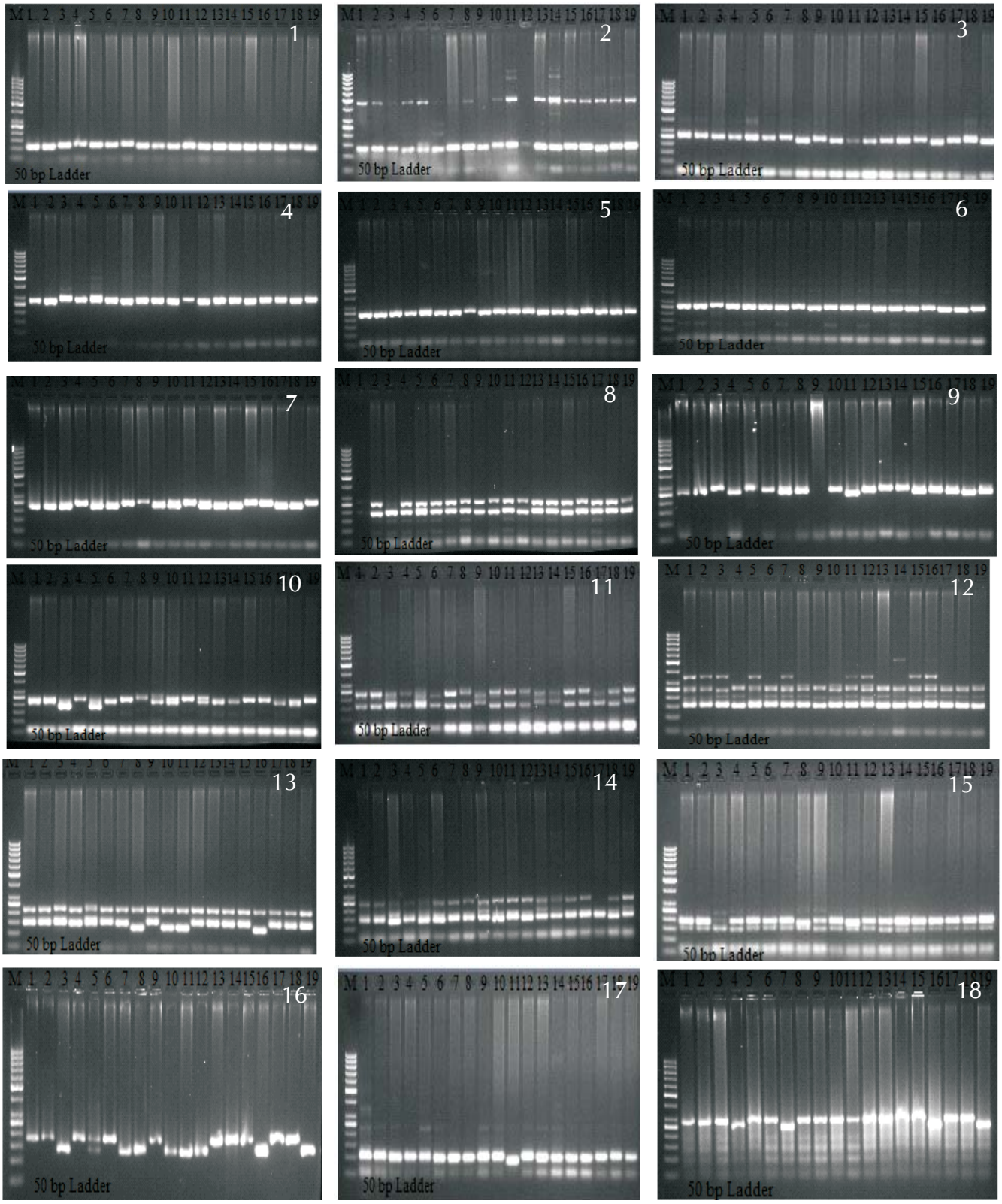

Figure 1-18: Typical SSR profiles obtained for 19 rice genotypes with primers (1) RM 23668, (2) RM 8303, (3)RM 23770, (4)RM 23778, (5)RM 23788, (6)RM 23805, (7)RM 23887, (8)RM 8300, (9)RM 23902, (10)RM 23915,(11) RM 23958(12)RM 215, (13) RM 257, (14) RM 23662, (15) RM 24013, (16) RM 910, (17)RM 7175 and (18) Sub-1 BC2

Table 2 : List of thirty four primers utilized for amplification of rice genomic DNA extracted from nineteen submergence tolerant genotypes used in the present study.

Locus	Chromo. some No	Primer sequence (5′-3′)	Repeat Motif	Annealing temp. (^{0}C)
RM 23668	9	(F) CAACTAGCCTACCGTGCAT(R) CGTGTTAAAGCAGCGAAACA	$(ACG)_{10}$	53
RM 23679	9	(F) AGTGCATGTTGAGCTTGTGG(R) ACCTGGCAATGAGAACGAGT	$(AGAA)_{10}$	54
RM 8303	9	(F) AGGGGAGAGGACACACACAC(R) GGATCCTCCTGCAAAATCAA	$(GGAGAGG)_4$	54
RM 23770	9	(F) GACCTTGTCCAGAGTGATTTTG(R) ATTTGAGAATAACTTTTCCTACTTCG	$(AAT)_{20}$	55
RM 23778	9	(F) ACACAGCCTAAAGGTGTTCTGAGC(R) GAGCTTCGGCCCTATAGTCTTCTC	$(ATAA)_{20}$	60
RM 23788	9	(F) ACCTTCACATAGCAGGGTTGAATC(R) ACTCTAAGCCCCTGGATAATCTGC	$(TAGG)_6$	58
RM 23805	9	(F) CACATAGTTTCCATGCTCGTTCAC(R) GGTAGAATCCATGACCGTCTCATC	$(TG)_{13}$	58
RM 23831	9	(F) TCATAGCGCACAGTTTCTTGAGTC(R) AGAGCGTTTGTGGGAAGTTTAATG	$(ATCT)_{11}$	57
RM 23843	9	(F) TCACAGACATAATTGTTTGGAGAAGG(R) CCAAAAGCTTTCATCTTTTTGTCC	$(TA)_{40}$	55
RM 23887	9	(F) ATCGATCGATCTTCACGAGG(R) TGCTATAAAAGGCATTCGGG	$(TATC)_6$	52
RM 8300	9	(F) GCTAGTGCAGGGTTGACACA(R) CTCTGGCCGTTTCATGGTAT	$(ACCATTAT)_3$	55
RM 23901	9	(F) CAGCTATTGAGACAACGCAAACAC(R) CAAAAATCATCTATTTTCGCAGGC	$(AT)_{45}$	56
RM 23902	9	(F) CGATTTGTTGTCAGCGTGAATTAG(R) GCATGCATGATTGAAGATTTGAAG	$(ATA)_{27}$	55
RM 23915	9	(F) TACATTGGAAGGAAATTCAGCTCC(R) CATGCAGATATGACCAAGAACCTG	$(AC)_{15}$	57
RM 23917	9	(F) CTCAGCTGTCTGTTCAGCTCTCAC(R) CTTTGGTGCTGAGGTAGGTATTGG	$(CA)_{12}$	60
RM 23922	9	(F) TGGAGGGAGTATCATTATTAGCCG(R) CTTGGATAGATTTGGTGGGATGAC	$(TG)_{14}$	58
RM 23928	9	(F) TGTGATATTTTTGAATCGTGGTGG(R) TGGCTATCCATAAGCTCGTTTCTC	$(TA)_{31}$	56
RM 23958	9	(F) GAGACAGATGTGTACGGTTTGGTG(R) TTGACAAGGGAATTGAAGGAGAAG	$(CT)_{15}$	58
RM23996	9	(F) GGATATCCATTAAGAAGAGGAAGTACA(R) ATCGCCTTTCGCATGTAAAC	$(TA)_{44}$	55
RM 24005	9	(F) TAAGCCCAAATCTTGCATGG(R) CATTGCGAGTGCGAGAGAT	$(AG)_{20}$	53
RM 24103	9	(F) ATTTTCCCCCTCCTTTTTGG(R) CCGGCACACAATGAATAGG	$(AC)_{17}$	53
RM 105	9	(F) GTCGTCGACCCATCGGAGCCAC(R) TGGTCGAGGTGGGGATCGGGTC	$(CCT)_6$	64
RM 215	9	(F) CAAAATGGAGCAGCAAGAGC(R) TGAGCACCTCCTTCTCTGTAG	$(GA)_{16}$	55
RM 257	9	(F) CAGTTCCGAGCAAGAGTACTC(R) GGATCGGACGTGGCATATG	$(CT)_{24}$	56
RM 316	9	(F) CTAGTTGGGCATACGATGGC(R) ACGCTTATATGTTACGTCAAC	$(GA)_{19}$	54
RM 23662	9	(F) GAGAGGACGATGGCACTATTGG(R) CGAGGAACTTGATTCGCATGG	$(GAAT)_{21}$	58
RM 24013	9	(F) TCCATCTTCCTCTCCTAGAGCTTCC(R) CTCCCTGTCCCGAGTTAGTGA	$(TGA)_{25}$	60
RM 910	9	(F) CTTTGGATTCAGGGGGA(R) AACTTGAAACGGAGGCAG	$(GAT)_{20}$	50
RM 7175	9	(F) ACAGTAAACGTGGTGCCTCC(R) AGAAGTAGCCTCGAGGACCC	$(GCAT)_{15}$	57
ART 5	9	(F) CAGGGAAAGAGATGGTGGA(R) TTGGCCCTAGGTTGTTTCAG	$(GAA)_{18}$	54
SC 3	9	(F) GCTAGTGCAGGGTTGACACA(R) CTCTGGCCGTTTCATGGTAT	$(CAG)_{16}$	55
RM 296	9	(F) CACATGGCACCAACCCTCC(R) GCCAAGTCATTCACTACTCTAG	$(GAC)_{14}$	57
Sub $1BC_2$	9	(F) AAAACAATGGTTCCATACGAGAC(R) GCCTATCAATGCGTGCTCTT	$(ACT)_{10}$	54
RM 23865	9	(F) TCATCCCATTCTCTTCCTCACC(R) CATACGGCCATACAAATGAACC	$(CT)_{10}$	55

Table 3 : Analysis of primer pairs used for the amplification of genomic DNA extracted from nineteen rice varieties

Primer	No of locus	Size of alleles (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*	*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*	*0.693*	*11*
RM 23887	*1*	*225-260*	*10*	*5*	*50*	*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*	*0.88*	*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*	*0.898*	*3*
RM 105	*1*	*103-131*	*9*	*3*	*33.33*	*0.864*	*0*
RM 215	*3*	*152-542*	*20*	*8*	*40*	*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*	*62.5*	*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*	*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*	*37.5*	*0.814*	*1*

Table 4 : Estimates of thirty four SSR primer pairs based Jaccard's similarity coefficients among nineteen rice varieties used in the Present study.

Variety	AnhHsung Seln (C11)	Hsung Teing	FR 13A	FR 13B	Karkati 87	Paiam	Kalonchi	Zobgui	Lunishree	Ranjbhoj	Singhara	Suraha	Sathi	Dihawan	Jadhan	Sarjoo 52	S-150	Kala Bunde
HsungTeing	0.293																	
FR 13A	0.013	0.04																
FR 13B	0.042	0.027	0.013															
Karkati 87	0.041	0.04	0.097	0.07														
Paiam	0.072	0.056	0.013	0.072	0.151													
Kalonchi	0.026	0.039	0.038	0.012	0.025	0.068												
Zobgui	0.042	0.041	0.013	0.057	0.013	0.088	0.083											
Lunishree	0.028	0.027	0.013	0.073	0.086	0.089	0.054	0.106										
Ranjbhoj	0.041	0.027	0.012	0.041	0.069	0.086	0.039	0.071	0.072									
Singhara	0	0	0.081	0.04	0.053	0	0.038	0.054	0.013	0.098								
Suraha	0.041	0.04	0.025	0.027	0.054	0.055	0.052	0.027	0.027	0.166	0.144							
Sathi	0.013	0.013	0.027	0.013	0.041	0.073	0.04	0.057	0.058	0.088	0.041	0.041						
Dihawan	0.027	0.027	0.013	0.013	0.041	0.057	0.068	0.042	0.057	0.027	0.026	0.027	0.196					
Jadhan	0.04	0.054	0.025	0.04	0.039	0.069	0.065	0.069	0.027	0.114	0.066	0.082	0.055	0.1				
Sarjoo 52	0.057	0.056	0.013	0.042	0.041	0.027	0.068	0.088	0.013	0.027	0.026	0.055	0.013	0.072	0.084			
S-150	0.014	0.013	0.013	0.014	0.042	0.028	0.07	0.043	0.014	0.013	0.013	0	0.092	0.043	0.056	0.125		
Kala Bunde	0.042	0.027	0	0.027	0.041	0.042	0.012	0.042	0.106	0.013	0.026	0.013	0.028	0.072	0.069	0.057	0.125	
Swarna Sub-1	0	0.013	0	0.027	0	0	0.039	0.013	0.027	0	0.026	0.026	0.042	0.013	0.012	0.041	0.028	0.027

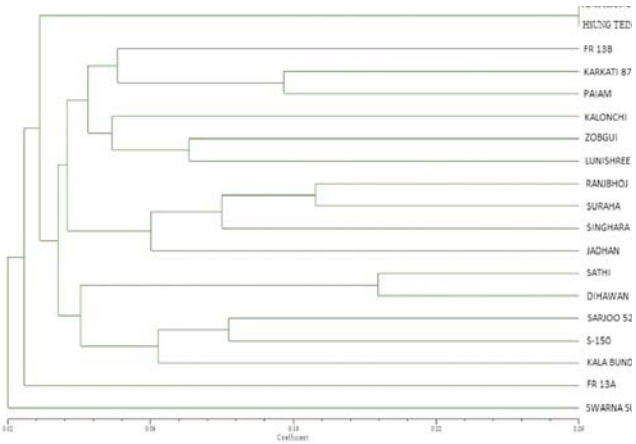

Figure 19: Dendrogram based on average Jaccard's similarity coefficient for thirty four SSR primer pairs among 19 rice genotypes.

of stutter bands was also detected in the present study. Stutter bands indicated the presence of minor products amplified in PCR that had lower intensity than the main allele and normally lacked or had extra units. Such bands were observed in the 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 1BC2 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 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 the 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 the repeat sequence.

Since, a change in the number of repeats leads to generation of allelic variants because of variation in the size of the SSR allele, the total repeat count of tri-nucleotide SSR loci was found to be associated with the number of alleles. Larger the repeat number involved in the SSR locus, the larger was the number of identified alleles. It can be observed that increase in number of allele with the repeat number of the microsatellites used and their relative distance from the centromere, which were not dependent on the motif of microsatellites (Biswas *et al*., 2012; Prathyusha *et al*., 2009).

Allelic diversity data was used to produce a dendrogram in order to elucidate the relationship among nineteen 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 a considerably greater extent of variation among the rice varieties under evaluation in the present study (Table 4). 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 that a very high degree of similarity exists between rice genotypes AnhHsungSeln (C 11) and HsungTeing, whereasFR 13A and Swarna Sub-1 found more diverse, may be used in breeding programme to generate more recombinants. Further the phenon line divided all other sub-clusters into mono-genotypic clusters (Fig. 19). Pattern of clustering and sub clustering indicated that the genotypes with distinct DNA profiles are likely to contain the novel genes and are likely to unique and agronomically useful, may be exploited for crop improvement.

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