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RESEARCH ARTICLE

Profiling of selected indigenous rice (*Oryza sativa* L.) landraces of Rarh Bengal in relation to osmotic stress tolerance

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Abstract A total of ten rare indigenous rice landraces of West Bengal were screened for germination potential and seedling growth under varying concentrations of sodium chloride (NaCl) and polyethylene glycol (PEG) solutions as osmotic stress inducing agents. Among the studied rice landraces *Kelas* and *Bhut Moori* showed highest degree of tolerance to induced osmotic stresses. Proline content of the studied lines was also determined. Genetic relationship among the studied rice landraces was assessed with 22 previously reported osmotic stress tolerance linked Simple Sequence Repeat (*SSR*) markers. The identified allelic variants in form of amplified products size (molecular weight) for each SSR marker were documented to find out allele mining set for the linked markers of the studied genotypes in relation to osmotic stress tolerance. A Microsatellite Panel was constructed for the different allelic forms (size of amplified products) of each used marker. Among 22 SSR markers, ten showed unique alleles in form of single specific amplified product for the studied four genotypes which can be used for varietal identification. Genetic relationship among the studied rice lines was determined and a dendrogram was constructed to reveal their genetic inter-relationship. Polymorphism Information Content (*PIC*) for each used marker was also calculated for the studied rice lines.

Keywords Indigenous rice landraces · Osmotic stress · Simple sequence repeat · Microsatellite panel · Polymorphism information content

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Introduction

Indigenous and wild rice landraces harbour a number of favourable genes that are not expressed in the phenotype of their parents (Tanksley and McCouch 1997; Moncada et al. 2001; Brondani et al. 2006; Septiningsih et al. 2003; Thomson et al. 2007) and have been used as donor parent for different stress tolerance in a number of hybridization programmes. Osmotic stresses including drought and salinity with other adverse conditions are frequently encountered as constraint to rice production in most of the rice growing regions of Rarh Bengal (the lateritic south western districts of West Bengal). Though genetic resources for tolerance of these stresses are available in wild and indigenous races, complexity of these traits hinder transfer of the tolerance genes into elite rice cultivars (Sundaram et al. 2007). QTLs for these traits have been identified and marker assisted breeding strategy have been used for specific QTL introgression into sensitive cultivars. The initial step for utilization of these traditional lines in breeding programme is proper physio-biochemical screening and also molecular marker based profiling in relation to different abiotic stress tolerance for confirmation of the traditional knowledge available from farmers. Most common way for physiological characterization is the study of different physiological parameters under different experimental

osmotic stresses, whereas for molecular profiling, different stress tolerance linked markers are used for genotyping of the studied lines. Though once (before 1970's) all the rice fields of Bengal were occupied by a good number of traditional lines (Rai 1999), in post green revolution decade most of such lines were replaced by high yielding rice lines (Deb 2005). The very few existing traditional rice lines of Rarh Bengal which are still maintained by few growers in some restricted pockets have been reported to be tolerant against various abiotic stresses (Deb 2005; Lodha et al. 2011). For present investigation, ten least cultivated indigenous rice landraces of Rarh Bengal were selected for which no earlier information is available. The objectives of present investigation were; (i) Profiling of selected rice lines possessing promising osmotic stress tolerance (as informed by growers) for different physiological parameters (ii) Genotyping with 22 osmotic stress tolerance linked SSR markers for determining the genetic relationship, and (iii) Preparation of a genetic databank (Microsatellite Panel) for proper varietal identification and utilization in marker assisted breeding programme.

Materials and methods

Plant materials used

The details of the rice landraces used in this study are given in Table 1. All of these are upland rice landraces of different districts of Rarh Bengal (Birbhum, Purulia and Bankura), the most drought prone area of South West Bengal (Fig. 1).

Fig. 1 Collection places of the selected landraces

Physiological screening under different osmotic stresses

For physiological screening, osmotic stresses were induced by two ways- salt stress induced by NaCl solution and drought stress induced by PEG 6000 solution. These stresses were imposed during seed germination and seedling growth of the selected landraces. In the first experiment, ten surface sterilized seeds (for each concentration) were grown at varying concentrations (100 mM, 200 mM and 300 mM) of NaCl for 15 days under salt stress as described by Santhi et al. (2010). For screening under drought stress, another set of ten surface sterilized seeds (for each concentration) was exposed to varying concentrations (19.6 %, 29.6 % and 36 %) of PEG that produce −0.5 MPa, −1.0 MPa and −1.5 MPa of osmotic stress respectively for 15 days as described by Agnihotri et al. (2007). Germination percentage, shoot and root length of germinated seedlings were monitored and recorded. For *in vitro* screening, ten surface sterilized seeds (for each concentration) were inoculated in Murashige and Skoog (MS) medium supplemented with three different concentrations (0.5 %, 1.0 % and 1.5 %) of NaCl and PEG solution individually (Tam and Lang 2003). Germination percentages were recorded for 15 consecutive days.

Proline estimation

To study the phenotypic variability in relation to osmotic stress tolerance of the studied lines, proline content was measured using a standardized protocol (Roy et al. 2009). Five hundred mg of leaf tissue from 30 days old normal rice plants

Table 1 Details of landraces used in this study

Sl. no.	Our accession no.	Name of landraces	Place of collection	Specific note collected from farmers
1.	VB155	Baid Dhusuri	Bankura	Upland and Drought tolerant
2.	VB157	Kalo Nuniya	Purulia	Upland, Drought tolerant
3.	VB158	Tulsimukul	Birbhum	Upland, Drought tolerant
4.	VB159	Noichi	Bankura	Upland, Drought tolerant
5.	VB160	Huggi Bhatta	Bankura	Upland, Drought tolerant
6.	VB161	Kelas	Bankura	Upland, Drought tolerant
7.	VB162	Bhut Moori	Bankura	Upland, Drought tolerant
8.	VB163	Kalodhan	Purulia	Upland, Early maturing, Drought escaping
9.	VB164	Rani Kajal	Birbhum	Upland, Drought tolerant
10.	VB165	Deula Bhog	Bankura	Upland, Drought tolerant

were collected and homogenized in a sterile mortar and pestle with 10 ml of 3 % sulphosalicyclic acid and centrifuged at 5,000 rpm for 10 min. Two ml of supernatant was added to 2 ml of glacial acetic acid and 2 ml of acid ninhydrin. The reaction mixtures were incubated at 100 °C in a water bath for 1 h followed by ice bath for 5 min. The cooled product was mixed vigorously with 4 ml of toluene in a separating funnel. Upper toluene layer was separated and absorbance was read at 520 nm.

Molecular screening

For molecular profiling, the studied landraces were genotyped with 22 reported SSR markers mapped within osmotic stress tolerance linked QTL loci from four different chromosomes.

DNA isolation

Genomic DNA of the studied rice genotypes was isolated following a pre-standardized protocol (Lodha et al. 2011) of our laboratory.

Marker selection for genotyping

Twenty two published drought and salt tolerance linked SSR markers were selected (Akagi et al. 1996; Panaud et al. 1996; Temnykh et al. 2000; McCouch et al. 2002) which were reported in different *indica* rice lines. Detailed information of these markers was collected from Gramene database (www.gramene.org), a freely available comparative web data resource for cereal crops. Details of these markers are given in Table 2. Primer sequences of all the selected markers were also subjected to Basic Local Alignment Search Tool (BLAST) in Gramene website for confirmation of their sequence complementarities in rice genome.

PCR amplification

For PCR amplification, 25 μl of reaction mixture containing 4 μl of genomic DNA (100 ng), 2.5 μl of 10X *Taq*-buffer, 1.0 μl of 50 mM $MgCl_2$, 0.25 μl of 2.5 mM dNTP, 1.0 μl of each of the forward and reverse primer (at a concentration of 10 pmole/μl), 0.1 μl (1 U) of 5 U/μl *Taq*-DNA polymerase was used. The amplification profile of the first cycle was 97 °C for 5 min, 55 °C for 2 min. For the next 35 cycles the temperature regime was 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min. The final extension was at 72 °C for 10 min (Lodha et al. 2011). The PCR reaction was performed in thermal cycler (M. J. Research, MC 013130) and all the PCR reagents were purchased from Fermentas Life Sciences, USA.

Table 2 Details of RM markers used in this study

Sl. no.	RM marker	Repeat motif	Primer seq. (forward)	Primer seq. (reverse)	Chr. no.
1.	RM493	(CTT)9	5′TAGCTCCAACAGGATCGACC3′	5′GTACGTAAACGCGGAAGGTG3′	1
2.	RM3412	(CT)17	5′AAAGCAGGTTTTCCTCCTCC3′	5′CCCATGTGCAATGTGTCTTC3′	1
3.	RM10745	(TATG)9	5′TGACGAATTGACACACCGAGTACG3′	5′ACTTCACCGTCGGCAACATGG3′	1
4.	RM140	(CT)12	5′TGCCTCTTCCCTGGCTCCCCTG3′	5′GGCATGCCGAATGAAATGCATG3′	1
5.	RM10764	(AT)28	5′AGATGTCGCCTGATCTTGCATCG3′	5′GATCGACCAGGTTGCATTAACAGC3′	1
6.	RM10772	(CTT)16	5′GCACACCATGCAAATCAATGC3′	5′CAGAAACCTCATCTCCACCTTCC3′	1
7.	RM315	(AT)4 (GT)10	5′GAGGTACTTCCTCCGTTTCAC3′	5′AGTCAGCTCACTGTGCAGTG3′	1
8.	RM223	(CT)25	5′GAGTGAGCTTGGGCTGAAAC3′	5′GAAGGCAAGTCTTGGCACTG3′	8
9.	RM212	(CT)67	5′CCACTTTCAGCTACTACCAG3′	5′CACCCATTTGTCTCTCATTATG3′	1
10.	RM162	(AC)20	5′GCCAGCAAAACCAGGGATCCGG3′	5′CAAGGTCTTGTGCGGCTTGCGG3′	6
11.	OSR2R	(CT)37	5′GGCTGCTCATCAGCTGCATGCG3′	5′TCGGCAGTGGTAGAGTTTGATCTGC3′	1
12.	RM209	(AC)20	5′ATATGAGTTGCTGTCGTGCG3′	5′CAACTTGCATCCTCCCCTCC3′	11
13.	RM287	(GA)21	5′TTCCCTGTTAAGAGAGAAATC3′	5′GTGTATTTGGTGAAAGCAAC3′	11
14.	RM10720	(TA)34	5′GCAAACGTCTACGTGAGAAACAAGC3′	5′GCATGTGGTGCCTTAACATTTGG3′	1
15.	RM10748	(AG)14	5′CATCGGTGACCACCTTCTCC3′	5′CCTGTCATCTATCTCCCTCAAGC3′	1
16.	RM10773	(AT)42	5′CCACCTATAGAAATAGGTCCATGC3′	5′TAGAACATGTCACCATCCAAGG3′	1
17.	RM10793	(ATAG)7	5′GACTTGCCAACTCCTTCAATTCG3′	5′TCGTCGAGTAGCTTCCCTCTCTACC3’	1
18.	RM8094	(AT)31	5′AAGTTTGTACACATCGTATACA3′	5′CGCGACCAGTACTACTACTA3′	1
19.	RM10825	(AAG)10	5′GGACACAAGTCCATGATCCTATCC3′	5′GTTTCCTTTCCATCCTTGTTGC3′	1
20.	RM10890	(TATC)5	5′GCTTCGGCTCTTCATTCACTGG3′	5′GCGATTATAGGAGCGCTATGTGG3′	1
21.	RM1287	(AG)17	5′GGAAGCATCATGCAATAGCC3′	5′GGCCGTAGTTTTGCTACTGC3′	1
22.	RM10852	(ATAG)5	5′GAATTTCTAGGCCATGAGAGC3′	5′AACGGAGGGAGTATATGTTAGCC3′	1

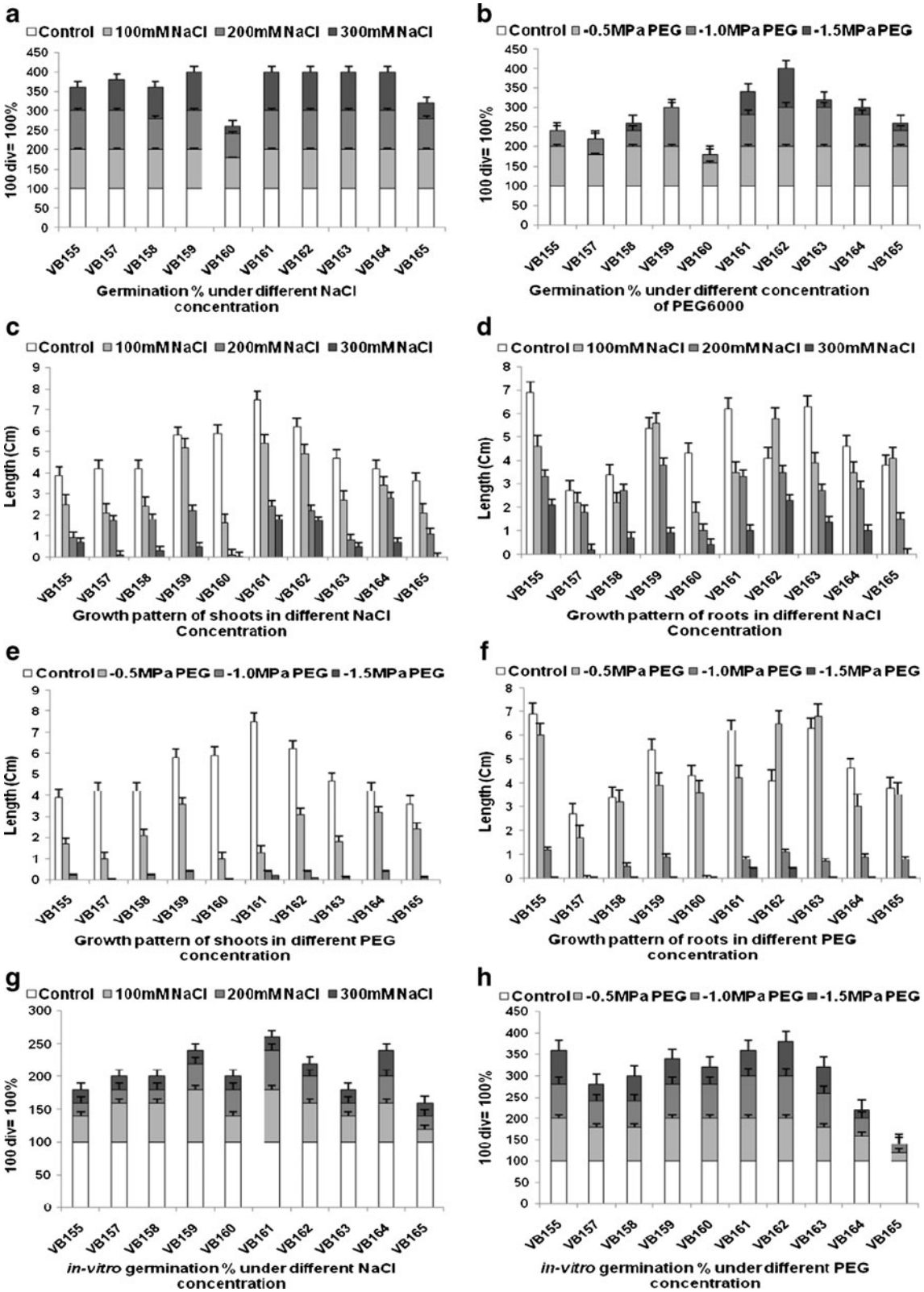

Fig. 2 Germination percentage and growth pattern of the studied rice landraces under different osmotic stresses (only final results are shown here instead of consicutive 15 days data to reduce complexity)

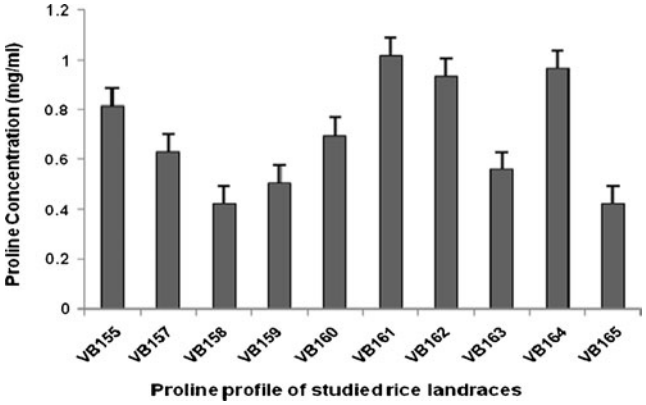

Fig. 3 Estimated proline profile of the studied rice landraces

Polymorphism screening, scoring and analysis of amplified products

The amplified products were resolved through 6 % native polyacrylamide gel following the protocol (Sambrook and Russell 2001) and documented using a gel documentation system (Perkin Elmer, Geliance 200 imaging system). The molecular weight of the amplified products (allelic variants) of the studied SSR markers was determined using analysis software (AlphaEaseFC 4.0, USA).

Marker scoring and data analysis

Individual alleles of all SSR markers were scored to prepare a 1/0 matrix based on presence (1) or absence (0). A pair-wise similarity coefficient matrix between all possible pairs of the rice genotypes were calculated from the 1/0 matrix using statistical software (SPSS 10.0) and a dendrogram was constructed using average linkage between groups. PIC values for each SSR marker was calculated by the following simplified formula: (Hwang et al. 2009)

$$PIC_i = 1 - \sum P_{ij}^2$$

Where, i=1 to n and P_{ij} is the frequency of j^{th} allele for the i^{th} band scored for a particular marker.

Results

Physiological screening under different osmotic stresses

All the studied landraces germinated at highest concentration (300 mM) of NaCl with varying potentiality. Noichi, Kelas, Bhut Moori, Kalodhan and Rani Kajal showed maximum germination percentage (100 %) in 300 mM of NaCl concentration within 15 days of treatment (Fig. 2a). On the other hand, six landraces (Tulsimukul, Kelas, Bhut Moori, Kalodhan, Rani Kajal and Deula Bhog) germinated at highest concentration (−1.5 MPa) of PEG, where, Bhut Moori showed highest germination percentage (100 %) within 15 days of treatment. Other four landraces (Baid Dhusuri, Kalo Nuniya, Noichi and Huggi Bhatta) showed germination up to −1.0 MPa PEG (Fig. 2b).

Among the studied rice landraces maximum, shoot growth (1.8 cm) was found in Kelas, where as Huggi Bhatta showed lowest shoot growth (0.05 cm) in highest concentration of NaCl (Fig. 2c). On the other hand, Bhut Moori showed highest root growth (2.3 cm) and Deula Bhog showed lowest root growth (0.05 cm) in highest concentration of NaCl (Fig. 2d).

In highest PEG concentration, maximum shoot growth (0.2 cm) noted in Kelas where as lowest shoot growth (1 cm in −0.5 MPa PEG concentration) noted in Kalo Nuniya and Huggi Bhatta (Fig. 2e). For root growth, Kelas and Bhut

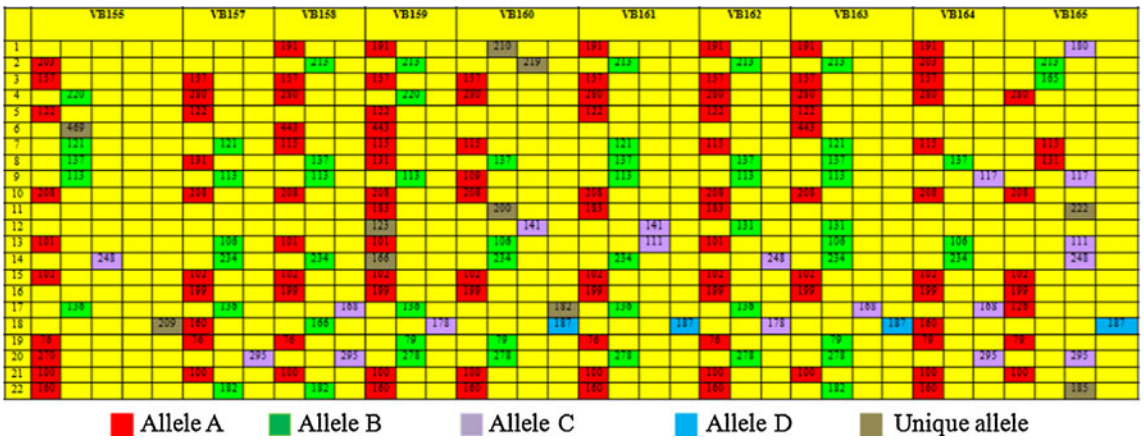

Fig. 4 Microsatellite panel of studied rice landraces against used 22 markers (here 1 to 22 represents all the used RM primers respectively as represented in Table 2)

Table 3 Similarity matrix of the studied rice landraces

Landraces	VB155	VB157	VB158	VB159	VB160	VB161	VB162	VB163	VB164	VB165
VB155	1.000									
VB157	0.366	1.000								
VB158	0.252	0.498	1.000							
VB159	0.306	0.444	0.420	1.000						
VB160	0.000	0.366	0.344	0.306	1.000					
VB161	0.397	0.789	0.631	0.677	0.599	1.000				
VB162	0.507	0.521	0.712	1.000	0.376	1.000	1.000			
VB163	0.288	0.521	0.833	0.446	0.570	0.873	0.720	1.000		
VB164	0.269	0.651	0.880	0.165	0.470	0.444	0.521	0.631	1.000	
VB165	0.152	0.470	0.444	0.306	0.324	0.495	0.570	0.288	0.581	1.000

Moori showed maximum growth (0.4 cm in highest PEG concentration), where as lowest (1.7 cm in −0.5 MPa PEG concentration) showed by Kalo Nuniya (Fig. 2f).

In case of *in vitro* screening, Kelas showed highest germination percentage in NaCl stress (Fig. 2g) and Bhut Moori showed in PEG stress (Fig. 2h) where as Deula Bhog showed lowest germination percentage in both NaCl and PEG stresses (Fig. 2g, h).

Proline estimation

Proline content of all the studied rice landraces are represented in Fig. 3. Among the studied lines, Kelas showed highest proline content (1.016 mg/ml) where as Tulsimukul showed lowest proline content (0.424 mg/ml).

Molecular screening

Different alleles, in form of variation in mol. wt. of each amplified products for each SSR marker against studied ten genotypes are given in a Microsatellite Panel (Fig. 4). The marker RM8094 produced maximum (five), while RM10764, RM162, RM10748, RM10773 and RM1287 generated only one allele across the studied ten lines. Among the used SSRs RM10720 showed highest range of allele size (166–248 bp).

A 1/0 matrix of the studied ten rice lines for 22 SSR markers was created on the basis of presence (1) or absence (0) of a specific band. From this 1/0 matrix similarity coefficients among the investigated lines were calculated using SPSS 10.0 (Table 3). In similarity coefficient matrix Noichi, Bhut Moori and Kelas, Bhut Moori showed highest similarity (1) where as Baid Dhusuri and Huggi Bhatta showed no similarity (0). On the basis of these values, a dendrogram (Fig. 5) was constructed using 'between group linkage' method using SPSS 10.0. The dendrogram showed two major clusters (Kelas, Bhut Moori, Noichi, Kalo Nuniya form one and Tulsimukul, Rani Kajal and Kalodhan form another). Rest three landraces (Deula Bhog, Huggi Bhatta and Baid Dhusuri) were joined to the super cluster formed by cluster one and two. Within the first cluster, Kelas and Bhut Moori are very much close to each other where as in the second cluster Tulsimukul and Rani Kajal are also close to each other. PIC values of all the

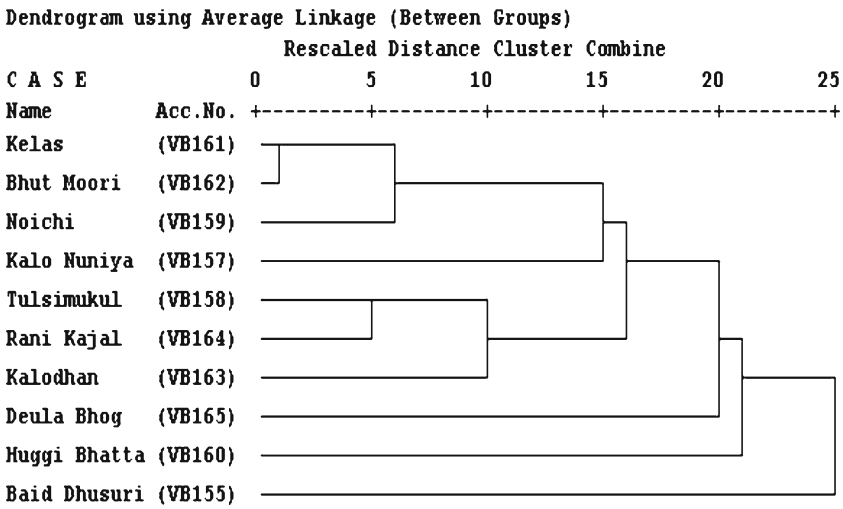

Fig. 5 Dendrogram of the studied rice landraces

Table 4 PIC values of selected RM markers

Sl. no.	Marker	PIC value
1.	RM493	0.62
2.	RM3412	0.59
3.	RM10745	0.18
4.	RM140	0.32
5.	RM10764	0.00
6.	RM10772	0.45
7.	RM315	0.48
8.	RM223	0.42
9.	RM212	0.46
10.	RM162	0.00
11.	OSR2R	0.34
12.	RM209	0.46
13.	RM287	0.64
14.	RM10720	0.54
15.	RM10748	0.00
16.	RM10773	0.00
17.	RM10793	0.64
18.	RM8094	0.74
19.	RM10825	0.42
20.	RM10890	0.58
21.	RM1287	0.00
22.	RM10852	0.54

studied 22 markers were calculated (Table 4) of which RM8094 showed highest PIC value (0.74).

Discussion

For the ten studied indigenous rice lines, there are no previous reports of physiological, biochemical and molecular investigation in relation to osmotic stress tolerance. Physiological profiling had shown that these landraces have osmotic stress tolerance property with varying potentiality. Two genotypes Kelas and Bhut Moori showed highest degree of tolerance in both the osmotic (NaCl and PEG solutions) stresses. Their high osmotic tolerance was also confirmed by high proline content, an osmolyte related to osmotic stress tolerance (Summart et al. 2011). Molecular profiling using osmotic stress tolerance linked markers for the studied landraces also showed high degree of diversification. The highest osmotic stress tolerance and closeness between the two studied lines was also confirmed through marker assisted genetic similarity study. Microsatellite Panel of the ten studied lines also revealed some genotype specific unique alleles which are significant not only for marker assisted selection related to osmotic stress tolerance but also for varietal identification and protection in Intellectual Property Rights (IPR) related issues. This work may form the preliminary base for investigation of the rest of the indigenous rice lines still existing in different isolated places under different stressful environments. For Rarh Bengal this study is significant as once this area harboured a good number of promising rice lines, most of which are lost except a very few which are still existing in some restricted pockets. This study also can be considered as one way to conserve the existing rice gene pool as well as popularization of the rice lines for further utilization. The characterized rice lines may be used in the future rice breeding programmes and also can be used to unlock the genetic potentials from the nature.

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