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RESEARCH ARTICLE

Screening of rice landraces for salinity tolerance at seedling stage through morphological and molecular markers

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Abstract The present investigation was carried out to evaluate 33 rice landrace genotypes for assessment of their salt tolerance at seedling stage. Growth parameters like root length, shoot length and plant biomass were measured after 12 days of exposure to six different levels of saline solution (with electrical conductivity of 4, 6, 8, 10, 12 or 14 dS m^{-1}). Genotypes showing significant interaction and differential response towards salinity were assessed at molecular level using 11 simple sequence repeats (SSR) markers, linked with salt tolerance quantitative trait loci. Shoot length, root length and plant biomass at seedling stage decreased with increasing salinity. However, relative salt tolerance in terms of these three parameters varied among genotypes. Out of the 11 SSR markers RM8094, RM336 and RM8046, the most competent descriptors to screen the salt tolerant genotypes with higher polymorphic information content coupled with higher marker index value, significantly distinguished the salt tolerant genotypes. Combining morphological and molecular assessment, four lanraces viz. *Gheus*, *Ghunsi*, *Kuthiahara* and *Sholerpona* were considered as true salt tolerant genotypes which may contribute in greater way in the development of salt tolerant genotypes in rice.

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Abbreviation

EC	Electric conductivity
QTL	Quantitative trait loci
SSR	Simple sequence repeats
UPGMA	Unweighted pair group method arithmetic average

Introduction

Rice (*Oryza spp.*) is an important cereal crop and is mainly used for human consumption. A 100 g of rice provides 345.0 kcal, 78.2 g of carbohydrates and 6.8 g of protein (Gopalan et al. 2007) inclusive of considerable amount of recommended Zinc and Niacin. Rice protein is biologically richest as its digestibility is very high (88 %). Rice provides almost 50–80 % of daily calorie intake amongst the poor class of the society. It's a staple food and cash crop for more than three billion people in the World (Ma et al. 2007). Asian farmers constitute about 92 % of the world's total rice producing group (Mitin 2009). In Asia 90 % of rice is produced by small farmers who are solely dependent on rice for their livelihood and food security (ANU 2006).

Salinity is the second most widespread soil problem in rice growing countries after drought and is considered as a serious limitation to increase rice production worldwide (Gregorio 1997). It causes yield reduction and also shrinks caloric and nutritional potential of agricultural products (Yokoi et al. 2002) causing leaf injury or death of plants, thus exceeding the capacity of salt compartmentalization in cytoplasm (Munns et al. 2006). Several studies indicated that rice is tolerant during germination, becomes very sensitive during

Table 1 Description of the land races of rice under study

Sl. No.	Accession No.	Name of the variety	Description
1.	PCS-1	Anjali	HYV, 85 days duration, released from Hazaribagh, India
2.	PLS-1	Annapurna	Local variety
3.	PLS-2	Ansapat	Local variety
4.	PCS-2	Barsha	HYV, Low land rice, 170 days, non-lodging
5.	PLS-3	Bhelkibagra	Local variety
6.	PLS-4	Chamarmuni	Local variety
7.	PLS-5	Dudheswar	Local variety
8.	PLS-6	Gheus	Local variety
9.	PLS-7	Ghunsi	Local variety
10.	PLS-8	Gobindabhog	Local variety
11.	PCS-3	Hiramoti	HYV, 115 days duration
12.	PLS-9	Kalobhog	Local variety
13.	PLS-10	Kaminibhog	Local variety
14.	PLS-11	Kanakchur	Local variety
15.	PLS-12	Kapur Dhul	Local variety
16.	PLS-13	Kataribhog	Local variety
17.	PLS-14	Kumrogor	Local variety
18.	PLS-15	Kuthiahara	Local variety
19.	PLS-16	Langalmura	Local variety
20.	PLS-17	Malabati	Local variety
21.	PLS-18	Marichshal	Local variety
22.	PLiS-1	Pankaj	Local improved variety
23.	PLiS-2	Patnai	Local improved variety
24.	PLS-19	Ramshal	Local variety
25.	PLS-20	Sabitapalui	Local variety
26.	PLS-21	Sadamota	Local variety
27.	PCS-4	Shatabdi	HYV, 105 days duration
28.	PLS-22	Sholerpona	Local variety
29.	PCS-5	Samelei	HYV, 145 days duration,
30.	PLS-23	Takshal	Local variety
31.	PLS-24	Talmugur	Local variety
32.	PLS-25	Thavallakanan	Local variety
33.	PLS-26	Tulaipanji	Local variety

early seedling stage (2–3 leaf stage), gains tolerance during vegetative growth stage, again becomes sensitive during pollination and fertilization and then becomes increasingly more tolerant at maturity (Lutts et al. 1995). As per the classification of tolerance to salinity, rice is within the sensitive division from 0 dS m^{-1} to 8 dS m^{-1} (Mass 1986). Quijano-Guerta and Kirk (2002) reported that the cheapest and easiest way to address the problem of salinity is through the development of a salt tolerant variety. For this, the foremost step is to screen the existing germplasms of paddy to identify the potential breeding materials. Screening at field level proved to be difficult due to soil heterogeneity, climatic factors and other environmental factors which may influence the physiological processes. Hence, screening under laboratory conditions is considered to be advantageous over field screening. However, conventional methods of screening for salt tolerance are not easy because of environmental effects and narrow sense heritability of salt tolerance (Gregorio 1997). This hinders the development of an accurate, rapid and reliable screening technique. In rice the screening can be done independently at its two salt sensitive stages but screening at seedling stage is a rapid method and based on simple criteria. In vegetative stage root length, shoot length and biomass have been proved as the potential indicators for screening of salt tolerance (Akbar et al. 1986; Jones 1986; Yeo and Flowers 1986; Flowers and Yeo 1995). It has also been reported that the assessment of the actual salt tolerance of the genotypes may be determined by comparisons of their biomass production only after a long growth period (Leland et al. 1994); which therefore serves as another criterion to evaluate the salt

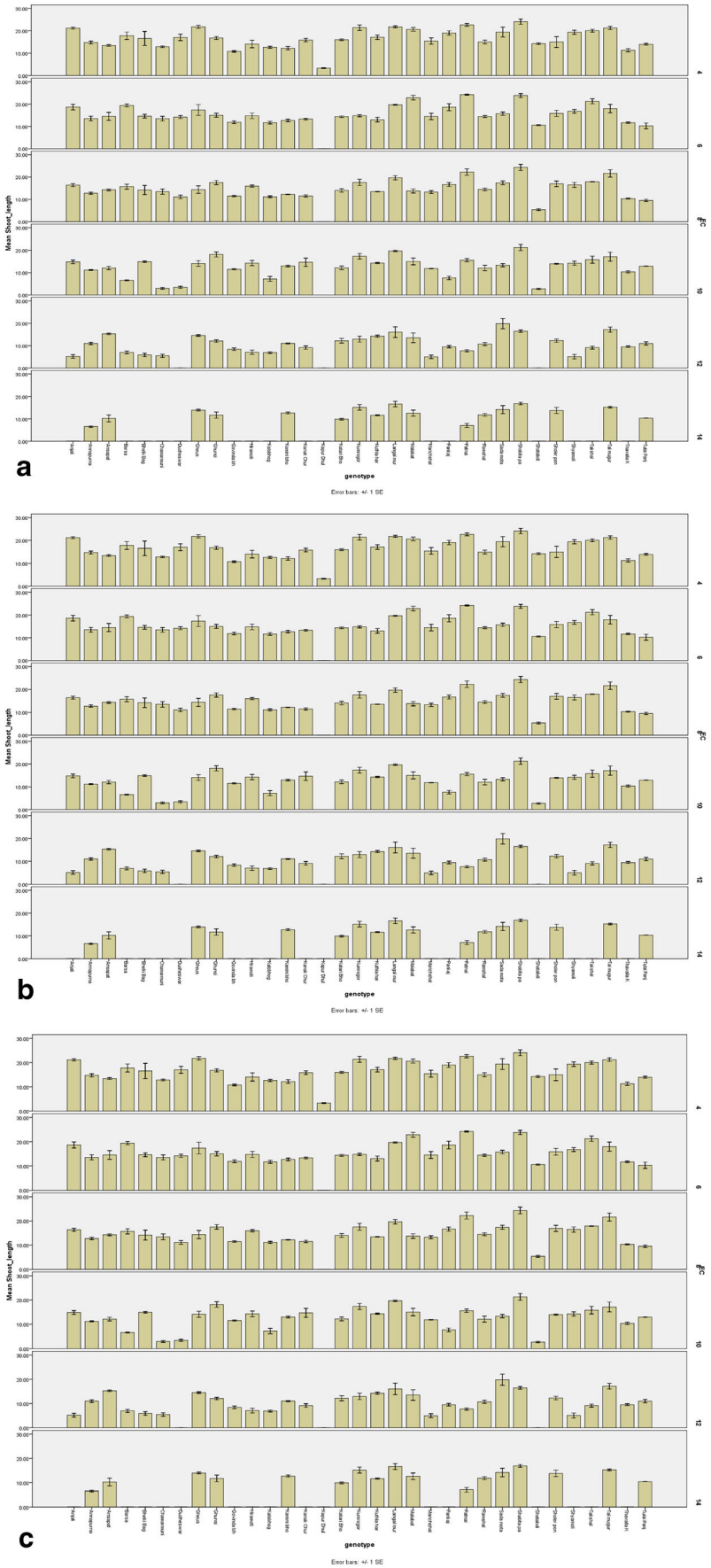

Fig. 1 Effect of different salinity level on **a.** shoot length, **b.** root length and **c.** plant biomass at 12 days after exposure to saline nutrient solution for 33 rice genotypes. *Error bars* represent standard error of the mean of three replicates

tolerance. However, the salt tolerance at early growth stages does not always correlate with that to the subsequent growth stages (Mass and Grieve 1994; Zeng et al. 2002; Ferdose et al. 2009). In this study, therefore, we focused on evaluating the potential of salt tolerance in rice genotypes at early growth stage i.e. at seedling stage. Screening of genotypes for salt tolerance at early stages may be important for screening salt tolerance as a there is considerable saving in time.

Use of molecular markers such as Restriction Fragment Length Polymorphism, Random Amplified Polymorphic DNA, Inter Simple Sequence Repeats, Simple Sequence Repeats (SSR) and Amplified Fragment Length Polymorphism (Karp et al. 1996) for screening genotypes is far more reliable than the morphological or physiological marker. Among the molecular marker technologies, microsatellite markers have been found to be effective in identification of genetic variability among rice cultivars (Garland et al. 1999, Islam 2004, Bhuiyan 2005). In this present global perspective when increase in rice production is a great task before us to meet our future demand, our present study aims to assess the performance of selected rice genotypes in terms of seedling growth parameters at varied salinity levels, to study selected morphological descriptors such as salinity index, to identify better molecular descriptors (SSR markers) useful for screening selected germplasms, and eventually to identify true salinity tolerant genotypes based on morphological and molecular descriptor for further breeding programs.

Materials and methods

The screening for salinity tolerance at seedling stage of the landrace genotypes of rice (listed in Table 1) was carried on the basis of morphological and molecular characterization. These 33 genotypes are being cultivated for several decades in southern part of West Bengal, the coastal saline belt of eastern India.

The viable (after Tri-phenyl tetrazolium test) and disinfected seeds (treated with 2 % (*w/v*) Bavistin for 8 h) were germinated in the glass plate. The plate was then placed on the flexi-glass tray and the whole set up was placed in a glass chamber, filled with 6 l of distilled water.

For a period for 4 days, after germination, seeds were kept in non-saline distilled water before they were exposed to saline nutrient solution of desired electrical conductivity (EC). The solutions with desired EC were prepared by dissolving NaCl in the nutrient solution (containing essential macro and micro elements). The EC of the solution was confirmed using EC meter (Model: Hanna HI 4321) and the pH was maintained at 5.0 (using pH meter Hanna HI 2211) for better availability of all the nutrients. The screening based on morphological response was carried out at six different EC levels viz. 4, 6, 8, 10, 12 and 14 dS m^{-1} (to identify genotypes for different levels of saline field situation, prevalent at coastal range of eastern India) following the criteria proposed by Gregorio et al. (1997).

Statistical analysis

The experiments were carried out in factorial completely randomized design (CRD) (Factor-1: Genotypes having 33 levels and Factor 2: Salinity having 6 levels) with three replications where each replication consisted of 10 samples. The data on morphological response of the seedlings due to saline exposure were collected after 12 days of treatment with respect to shoot length, root length and biomass. The evaluation was done using modified Standard evaluating score (SES) in rating the visual salt injury at seedling stage following the method proposed by Gregorio et al. (1997). The statistical analyses of morphological data were done using SPSS 16.0 for Windows and XLSTAT-2012 statistical software. Clusters of genotype were identified by using sequential multivariate statistical techniques cluster analysis (Ding 2004). Euclidian distance and Unweighted Pair Group Method Arithmetic Average (UPGMA) was considered for clustering.

Molecular screening with SSR markes linked salt QTL and data analysis

Genomic DNA isolation was carried out with 250 mg tender leaf tissue using CTAB method (Doyle and Doyle 1990) (buffer containing 100 mMTris, 1.4M NaCl, 20 mM EDTA) with few modifications. The concentration of DNA was determined by a UV–vis spectrophotometer (Perkin Elmer, Germany) and quality of genomic DNA was checked following electrophoresis on 0.8 % agarose gel. The quantified DNA was then diluted to a final concentration of 10 ng μl^{-1}.

The 25 μl optimized polymerase chain reaction (PCR) mixture contained 50 ng template DNA, 2.5 μl 10X Taq polymerase assay buffer, 2 mM $MgCl_2$, 200 μM of each dNTP, 0.1 μM of each primer and 0.5 U Taq DNA polymerase in HPLC grade sterile water. The PCR amplification comprised of an initial denaturation at 95 °C for 5 min followed by 35 cycles of 30 s at 95 °C, 60 s at 40–55 °C and 60 s at 72 °C, and final extension at 72 °C for 5 min, carried out using Eppendorf PCR system (Eppendorf Mastercycler Gradient, Germany). The procedure of SSR analysis was done following the method proposed by Yang et al. (1994) with modifications using SSR marker associated with salt tolerance at seedling stage. A total of 11 pairs of primers, linked with salt tolerance quantitative trait loci, were employed (Source: http://www.gramene.org/markers/microsat/).

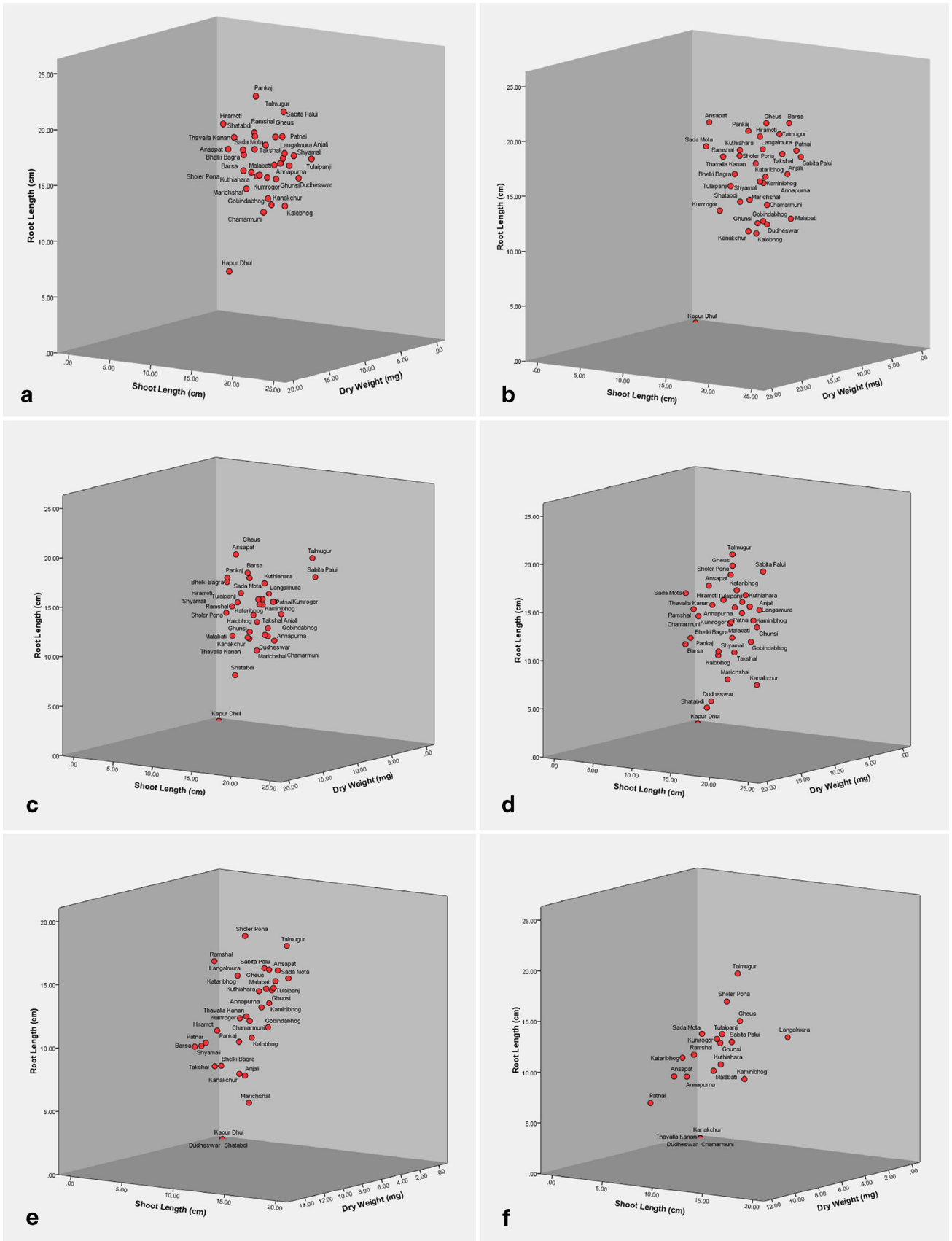

Fig. 2 Distribution of genotypes under study in 3-dimensitonal space of morphological salinity indices at seedling stage at **a.** EC-4, **b.** EC-6, **c.** EC-8, **d.** EC-10, **e.** EC-12 and **f.** EC-14 salinity levels

Aliquots of 4.0 µl amplified PCR products, along with DNA ladder were resolved by electrophoresis on 10 % Polyacrylamide gel in 1X TBE buffer, stained with ethidium bromide.

Scoring of amplified fragments

Gels were scanned with the Multi Doc-IT Digital Imaging Systems (S/N 032310-004) and analyzed using NTSYSPC software (version 2.01). The size of the PCR products were compared to the molecular size standard 50 bp DNA ladder. The well-separated and consistently reproducible, amplified DNA fragments (bands) were scored as being present (1) or absent (0) for SSR markers in data sheet to form a [1, 0] matrix. Then data were analyzed and dendrogram were generated with Unweighted Pair Group Method Arithmetic Average (UPGMA) algorithm (Rohlf 1993). The efficiency of primers were assessed on the basis of Polymorphic Information Content (PIC = $1-\sum p_i^2$, where p_i is the frequency of the ith allele (Smith et al. 1997) value and marker index

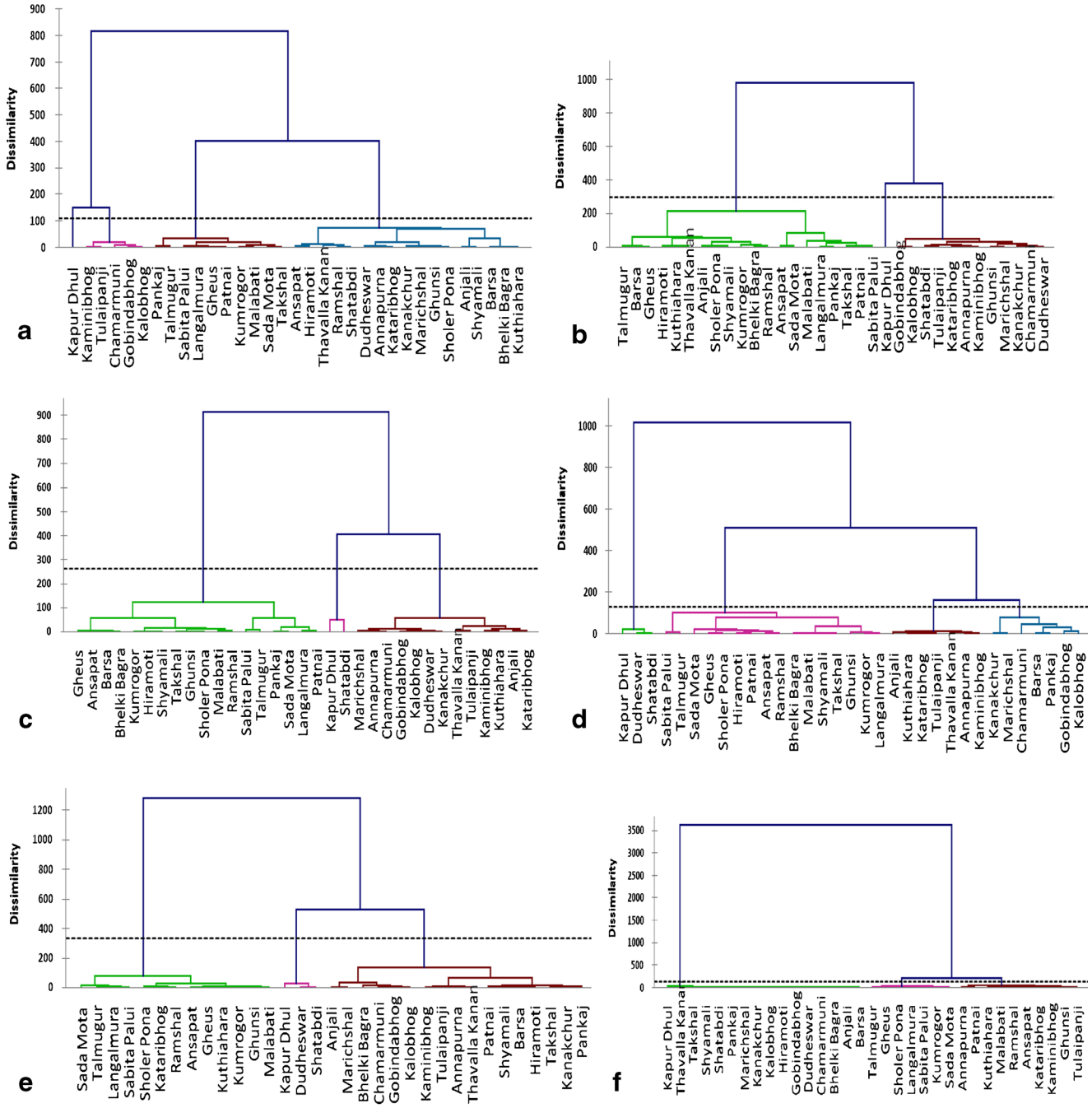

Fig. 3 Clusters of genotypes under study using Euclidian distance matrix at **a.** EC-4, **b.** EC-6, **c.** EC-8, **d.** EC-10, **e.** EC-12 and **f.** EC-14

Table 2 Correlation matrix among the variables at different level of salinity

Variables	EC	Shoot Length	Plant Biomass	Tolerance Index
Root length	6	0.709**	0.743**	−0.005
	8	0.791**	0.785**	−0.215
	10	0.720	0.746	−0.624
	12	0.919	0.859	−0.740
	14	0.970	0.931	−0.878
Shoot length	6		0.749**	−0.057
	8		0.792**	−0.276
	10		0.816**	−0.584**
	12		0.871**	−0.729**
	14		0.937**	−0.889**
Plant biomass	6			0.001
	8			−0.139
	10			−0.667**
	12			−0.655**
	14			−0.915**

(** different from 0 with a significance level alpha=0.01)

[MI = PICX Proportion of polymorphic bands × average number of loci per assay unit] (Powell et al. 1996).

Results and discussion

Response of seedling growth at different salinity level

The distribution for shoot length, root length and plant biomass in genotypes under study showed wide fluctuations across six different salinity level. All three seedling growth parameters were found to be minimum in case of *Kapur Dhul* at 4 dS m $^{-1}$ after which no germination was found for this landrace. The seedling root length ranged from 4.57 to 24.23 at 4 dS m $^{-1}$, 0 to 22.18 at 6 dS m $^{-1}$, 0 to 20.62 at 8 dS m $^{-1}$, 0 to 20.33 at 10 dS m $^{-1}$, 0 to 19.22 at 12 dS m $^{-1}$ and 0 to 20.91 at 14 dS m $^{-1}$. Maximum root length at 4, 6, 8, 10, 12 and 14 dS m $^{-1}$ were found in *Pankaj*, *Ansapat*, *Ansapat* + *Talmugur*, *Talmugur*, *Sholerpona* + *Talmugur*, and *Talmugur* respectively (Fig. 1b). The shoot length ranged from 3.28 to 24.08 at 4 dS m $^{-1}$, 0 to 23.80 at 6 dS m $^{-1}$, 0 to 24.33 at 8 dS m $^{-1}$, 0 to 21.26 at 10 dS m $^{-1}$, 0 to 19.83 at 12 dS m $^{-1}$ and 0 to 16.89 at 14 dS m $^{-1}$. *Sadamota* exhibited maximum shoot length at all EC level except EC 10 where *Sabitapani* recorded maximum shoot length (Fig. 1a). The plant biomass ranged from 2.50 to 19.00 at 4 dS m $^{-1}$, 0 to 20.67 at 6 dS m $^{-1}$, 0 to 17.83 at 8 dS m $^{-1}$, 0 to 17.00 at 10 dS m $^{-1}$, 0 to 14.33 at 12 dS m $^{-1}$ and 0 to 12.00 at 14 dS m $^{-1}$. In 4, 6, 10 and 12 dS m $^{-1}$ *Sabitapalui* recorded highest plant biomass. Both *Pankaj* and *Patnai* exhibited maximum plant bimass at 8 dS m $^{-1}$ and at 14 dS m $^{-1}$ *Sabitapalui*, *Sadamota* showed highest plant biomas (Fig. 1c). Shoot length, root length and plant biomass at seedling stage decreased with increasing salinity. However, relative salt tolerance in terms of these three parameters varied among genotypes (Fig. 1). Figure 2 represents the distribution of morphological salinity indices for the genotypes under study in three-dimensional space at seedling stage in all the ECs studied. From this figures it is clear that with the increasing salinity, morphological salinity indices of seedling stage are able to discriminate germplasm under study to greater extent. The low salinity treatment (6 dS m $^{-1}$) reduced these parameters of seedling to a lesser degree than moderate (8 and 10 dS m $^{-1}$) and high salinity treatments (12 and 14 dS m $^{-1}$). Except *Kapur Dhul* no genotypes showed susceptibility up to 10 dS m $^{-1}$. At 12 dS m $^{-1}$ *Dhudheswar* did not survive. From Figs. 1 to 3, comparing the performance of other genotypes with *Talmugur* (tolerant check) and *Shatabdi* (susceptible check) it can be clearly stated that the genotypes namely *Solerpona*, *Sabitapalui*, *Sadamota*, *Ramshal*, *Langalmur*, *Kataribhog*, *Gheus*, *Ghunsi*, *Ansapat* and *Kumargor* may be utilized as tolerant breeding stock for further crop improvement programme.

Most of the genetic variations against environmental stresses including salt stress are controlled by a large number of genes, each with small effects, scattered all over the genome. The effects of salinity on rice have been reported to reduce seed germination, decrease growth and survival of seedlings (Lutts et al. 1995). The significant reduction in seedling growth by salinity may be due to toxic effects of NaCl and unbalanced nutrient uptake by the seedlings. These deleterious effects of salinity may result in a significant decrease in photosynthesis rate and increase in respiration rate of seedlings that leads to a shortage of assimilate to the developing organs and may slow down growth or stop it entirely (El-Hendawy et al. 2005). Interestingly, the variations of the salt tolerance indices among genotypes were less at low salinity concentration than at high salinity concentration (Fig. 1).

Table 3 Multiple correlation coefficients (R) between Tolerance index (dependent variable) and shoot length, root length and plant biomass (independent variables) at different salinity level

Parameters	Electrical Conductivity (dS m $^{-1}$)				
	6	8	10	12	14
R- value	0.09^{NS}	0.31 ^{NS}	0.693**	0.750**	0.920**
R^2-value	0.008	.096	0.481	0.563	0.846

Correlation and regression analysis

The relationships between the seedling parameters and salt tolerance at 6 different salinity levels expressed in terms of

Table 4 Regression coefficients for shoot length, root length and plant biomass (independent variables) at different salinity level and constant (dependent variable is tolerance index)

Parameters	Regression coefficients				
	EC 6	EC 8	EC 10	EC 12	EC 14
Constant	2.385^{NS}	3.875**	5.885**	7.119**	8.586**
Root Length (cm)	0.036^{NS}	−0.099 ^{NS}	−0.278 ^{NS}	−0.465 ^{NS}	−0.013
Shoot Length (cm)	−0.144^{NS}	−0.400 ^{NS}	−0.025 ^{NS}	−0.327 ^{NS}	−0.250
Plant biomass (mg)	0.082^{NS}	0.256 ^{NS}	−0.439 ^{NS}	0.030 ^{NS}	−0.669**

electrical conductivity (EC) i.e. EC 4 (control), 6, 8, 10, 12 and 14 dS m $^{-1}$ are presented and discussed here. A significant positive correlation was found between root length with shoot length and dry weight at lower salinity levels (6 and 8 dS m $^{-1}$), however, with the increase of salinity no such significant association was found. Highly positive correlation between (0.75 and 0.93) shoot length and plant biomass, irrespective of salinity, have been observed. But root length and tolerance index (Standard Evaluation Score) showed insignificant relation which indicates that root length may not be a good descriptor of salinity tolerance. Though shoot length and plant biomass at lower salinity (6 and 8 dS m $^{-1}$) showed insignificant correlation with tolerance index but with increase in salinity, significant negative correlation (−0.58 to −0.92) was observed (Table 2). Negative association indicates higher the plant biomass and/or shoot length lower the tolerance index (Standard evaluation score) meaning more tolerance of genotypes. This finding may help to conclude that shoot length and plant biomass might be the better descriptors of salinity tolerance of genotypes in comparison to root length. Thus for screening the tolerant genotypes at seedling stage, its shoot length and biomass may be considered as selection criteria. The multiple correlation coefficient (R) between tolerance index (dependent variable) and shoot length, root length and plant biomass (independent variables) at different salinity level also showed similar results. All three independent variables also showed significant positive association with tolerance index. The value of R^2 gives the percentage of variation of tolerance index explained by independent variables. From the present findings it was observed that with the increase of salinity from 10 to 14 dS m $^{-1}$ the explained variation increased from 48 to 84.6 % (Table 3). From Table 4 the multiple regression line for each salinity level could be formed using the constant value and regression coefficients for root length, shoot length and plant biomass. From this finding it may be concluded that to screen true tolerant genotypes, the same should be screened at higher salinity.

Table 5 Cluster mean of different salinity indices in the clusters formed at different salinity level using Euclidian distance matrix

EC	Cluster	Root Length	Shoot Length	Plant Biomass	SES
4	1	17.346	15.942	12.475	NA
	2	13.464	12.486	6.334	NA
	3	20.367	21.196	17.689	NA
	4	4.570	3.280	2.500	NA
6	1	17.740	15.820	12.560	1.000
	2	13.030	13.470	7.330	1.000
	3	0.000	0.000	0.000	1.000
8	1	12.705	12.379	8.515	3.308
	2	16.868	17.287	14.198	2.778
	3	2.915	2.700	1.915	3.000
10	1	14.987	12.683	8.190	3.286
	2	15.994	15.566	13.180	2.750
	3	9.579	8.927	7.181	4.714
	4	1.743	2.080	1.277	5.000
12	1	9.775	7.875	6.801	4.882
	2	16.455	14.405	11.351	2.846
	3	0.000	0.000	0.000	7.000
14	1	0.000	0.000	0.000	8.625
	2	10.940	10.456	8.913	3.800
	3	16.229	15.119	10.017	3.000

At the saline condition, correlations between salt tolerance and plant height, plant biomass and root length were inverse and significant, which implied that salt tolerant genotypes (having lower salt tolerance score) exhibited higher plant height and total dry matter. Peng et al. (1999) reported that increasing plant height would allow greater biomass production.. The highly significant correlations between SES and Shoot length and plant biomass at the seedling stage further confirmed the importance of these parameters as useful selection criteria for screening the salt tolerance in terms of grain yield among genotypes.

Cluster analysis

Hierarchical clustering using Euclidean Distance as distance measure was done for grouping the germplasms suitably. On the basis of dendrogram (Fig. 3) constructed by UPGMA three to four clusters were formed at different level of salinity. At 4 dS m $^{-1}$ and 10 dS m $^{-1}$ four clusters were constructed. In the rest of the salinity level three clusters were formed. Class variance in different cluster at different salinity level ranged between 0 and 46.9. At 4 dS m $^{-1}$ in cluster 1, 17 genotypes were included, the same

Table 6 Rankings of genotypes for their relative salt tolerance in terms of shoot length, root length and plant biomass in a cluster analysis Euclidian distance matrix

Genotypes	Salinity levels						Sum[a]	Genotype Ranking[b]	Tolerance Degree[b]
	EC4	EC 6	EC8	EC10	EC12	EC14			
Anjali	2	1	2	2	2	3	12	7	Moderate
Annapurna	2	2	2	2	2	2	12	7	Moderate
Ansapat	2	1	2	1	1	2	9	4	Tolerant
Barsa	2	1	1	3	2	3	12	7	Moderate
Bhelkibagra	2	1	1	1	2	3	10	5	Moderate
Thavallakanan	2	1	2	2	2	3	12	7	Moderate
Dudheswar	2	2	2	4	3	3	16	11	Susceptible
Ghunsi	2	2	1	1	1	2	9	4	Tolerant
Hiramoti	2	1	1	1	2	3	10	5	Moderate
Kanakchur	2	2	2	3	2	3	14	9	Susceptible
Kataribhog	2	2	2	2	1	2	11	6	Moderate
Kuthiahara	2	1	2	2	1	2	10	5	Moderate
Marichshal	2	2	2	3	2	3	14	9	Susceptible
Ramshal	2	1	1	1	1	2	8	3	Tolerant
Shatabdi	2	2	3	4	3	3	14	9	Susceptible
Sholerpona	2	1	1	1	1	1	6	1	Tolerant
Shyamali	2	1	1	1	2	3	10	5	Moderate
Chamarmuni	3	2	2	3	2	3	15	10	Susceptible
Gobindabhog	3	2	2	3	2	3	15	10	Susceptible
Kalobhog	3	2	2	3	2	3	15	10	Susceptible
Kaminibhog	3	2	2	2	2	2	13	8	Moderate
Tulaipanji	3	2	2	2	2	2	13	8	Moderate
Gheus	1	1	1	1	1	1	6	1	Tolerant
Kumrogor	1	1	1	1	1	1	6	1	Tolerant
Langalmura	1	1	1	1	1	1	6	1	Tolerant
Malabati	1	1	1	1	1	2	7	2	Tolerant
Pankaj	1	1	1	3	2	3	11	6	Moderate
Patnai	1	1	1	1	1	2	7	2	Tolerant
Sabitapalui	1	1	1	1	1	1	6	1	Tolerant
Sadamota	1	1	1	1	1	1	6	1	Tolerant
Takshal	1	1	1	1	2	3	9	4	Tolerant
Talmugur	1	1	1	1	1	1	6	1	Tolerant
Kapur Dhul	4	3	3	4	3	3	20	12	Susceptible

[a] Sums were obtained from the cluster group rankings by adding the ranking numbers at the six salt levels in each genotype. [b] Genotypes were finally ranked based on the sums with the smallest sum being the most relatively tolerant.

in cluster 2 were five genotypes and in cluster 3 ten genotypes including *Talmugur* were observed. Whereas, *Shatabdi* with other 16 genotypes were grouped into cluster 1. The similar trend of observation was also found up to 10 dS m^{-1}.

In case of higher salinity i.e. 12 dS m^{-1} and 14 dS m^{-1} the grouping of genotypes in different clusters were found more or less similar. The tolerant check *Talmugur* with other 12 genotypes were found in cluster 2 at 12 dSm $^{-1}$ however, with other six genotypes it was found in the same cluster but at 14 dS m^{-1}. The cluster pattern at 14 dS m^{-1} indicates the six genotypes mainly *Gheus*, *Kumrogor*, *Langalmura*, *Shabitapalui*, *Sadamota* and *Sholerpona* to be highly salt tolerant genotypes. The genotypes *Ansapat*, *Ghunsi*, *Kataribhog*, *Kuthiahara*, *Malabati* and *Ramshal* were also grouped with *Talmugur* at 12 d Sm $^{-1}$ which indicates the higher limit of tolerance of the said genotypes. Thus from the cluster analysis particularly from 12 to 14 d Sm $^{-1}$ it can be concluded that the before mentioned genotypes may be utilized for further breeding programme against salinity tolerance of rice.

Table 7 Number of alleles, amplicon size, polymorphism information content (PIC) and marker index (MI) value of SSR marker for 33 rice genotypes

Marker	Frequency of major allele	Number of alleles	PIC value	Amplicon size range (bp)	Marker index
RM 8094	0.24	8	0.82	80–250	1.92
RM 493	0.24	5	0.78	200–300	0.73
RM 3412	0.24	5	0.75	80–270	0.93
RM 8095	0.45	5	0.67	90–250	1.16
RM 7075	0.21	5	0.71	125–250	1.01
RM 336	0.21	7	0.84	135–250	1.11
RM 253	0.58	4	0.69	125–180	1.17
RM 436	0.42	3	0.55	80–125	0.77
RM 8046	0.18	6	0.82	125–225	1.56
RM 8053	0.33	5	0.77	95–315	1.36

Cluster means for: root length, shoot length, plant biomass and Standard Evaluation Score (SES) at different levels of salinity, represented in Table 5. Maximum root length (20.36 cm), Shoot length (21.19 cm) and Plant biomass (17.69 mg) were found in cluster 3 at 4 dS m^{-1}. In case of higher salinity maximum root length (16.229 cm), shoot length (15.119 cm) and plant biomass (10.02 mg) were found in cluster 3 at 14 d Sm^{-1}. But at 12 dS m^{-1} they were found maximum at cluster 2. Both cluster 2 and cluster 3 at 12 dS m^{-1} and 14 dS m^{-1} respectively, having *Talmugur* as their member, indicates the tolerant group of genotypes. Inter cluster distance at different salinity levels are presented in Table 5. At 4 dS m^{-1} between cluster 4 and cluster 3; at 6 dS m^{-1} between cluster 3 and 1; at 8 dSm^{-1} between cluster 3 and 2; at 10 dS m^{-1} between cluster 4 and cluster 2; at 12 dS m^{-1} between cluster 3 and cluster 2 and at 14 dS m^{-1} cluster 3 and cluster 1 maximum inter cluster distance were observed. Higher the inter cluster difference between two clusters mean higher chance of getting genetic gain from the hybridization between the members of those two clusters.

To rank the salt tolerance of genotypes at the vegetative stage based on shoot growth parameters, genotypes were divided into four cluster groups at 4 and 10 dS m^{-1} and three cluster groups at high salinity by using Euclidian distance matrix (Table 6). The cluster group rankings were obtained from the cluster means (Table 5) in the order from the highest to the lowest clusters. Sums were obtained from the cluster group rankings by adding the ranking numbers at the six salt levels in each genotype. Genotypes were finally ranked based on the sums with the smallest sum being the most relatively tolerant. The most salinity tolerant germplasms were *Sholerpona*, *Takshal*, *Sada Mota*, *Sabita Palui*, *Patnai*, *Langalmura*, *Kumrogor*, *Gheus*, *Ramshal* and *Ansapat*. During the trials, visual damage on seedling leaves was not obvious at 4 dS m^{-1} although chlorosis was occasionally observed on some plants. With increase of salinity, visual damage was serious; chlorosis and leaf rolling were observed on all plants expressed as SES in Table 5.

Salt tolerance among rice genotypes was evaluated in this study using cluster analysis. As pointed out by Zeng et al.

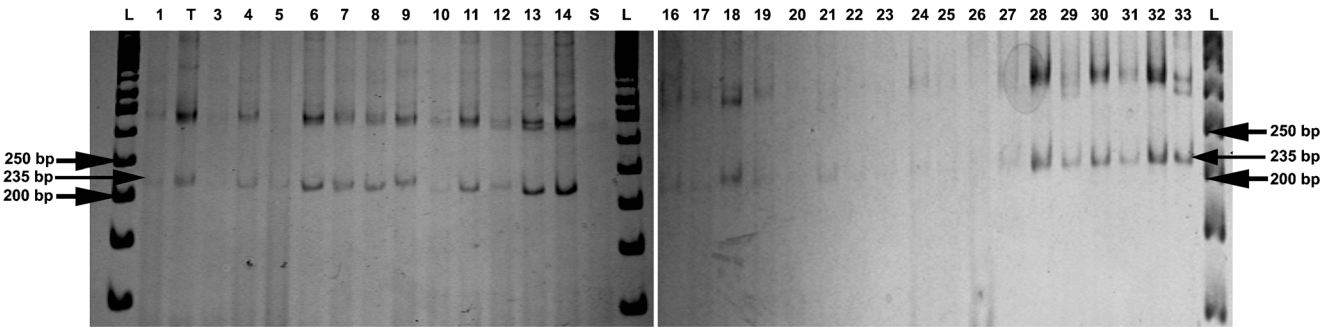

Fig. 4 Polyacrylamide gel electrophoresis of SSR fragments (generated by primer RM 8094) of 33 rice genotypes under study where Lane L-50 bp ladder, 1=*Sholerpona*, T=Tolerant check (*Talmugur*), 3=*Kalobhog*, 4=*Sabitapalui*, 5=*Kumrogor*, 6=*Taksal*, 7=*Gheus*, 8=*Kuthiahara*, 9=*Barsa*, 10=*Ansapat*, 11=*patnai*, *Pankaj*, 13=*Bhelkibagra*, 14=*Sadamota*, 16=*Chamarmuni*, 17=*Gobindabhog*, 18=*Dudheswar*, 19=*Langalmura*, 20=*Kapur Dhul*, 21=*Tulaipanji*, 22=*Kanakchur*, 23=*Kanakchur*, 24=*Thavallakanan*, 25=*Shyamali*, 26=*Hiramoti*, 27=*Annapurna*, 28=*Anjali*, 29=*Malabati*, 30=*Kaminibhog*, 31=*Ramshal*, 32=*Marichshal* and 33=*Kataribhog*

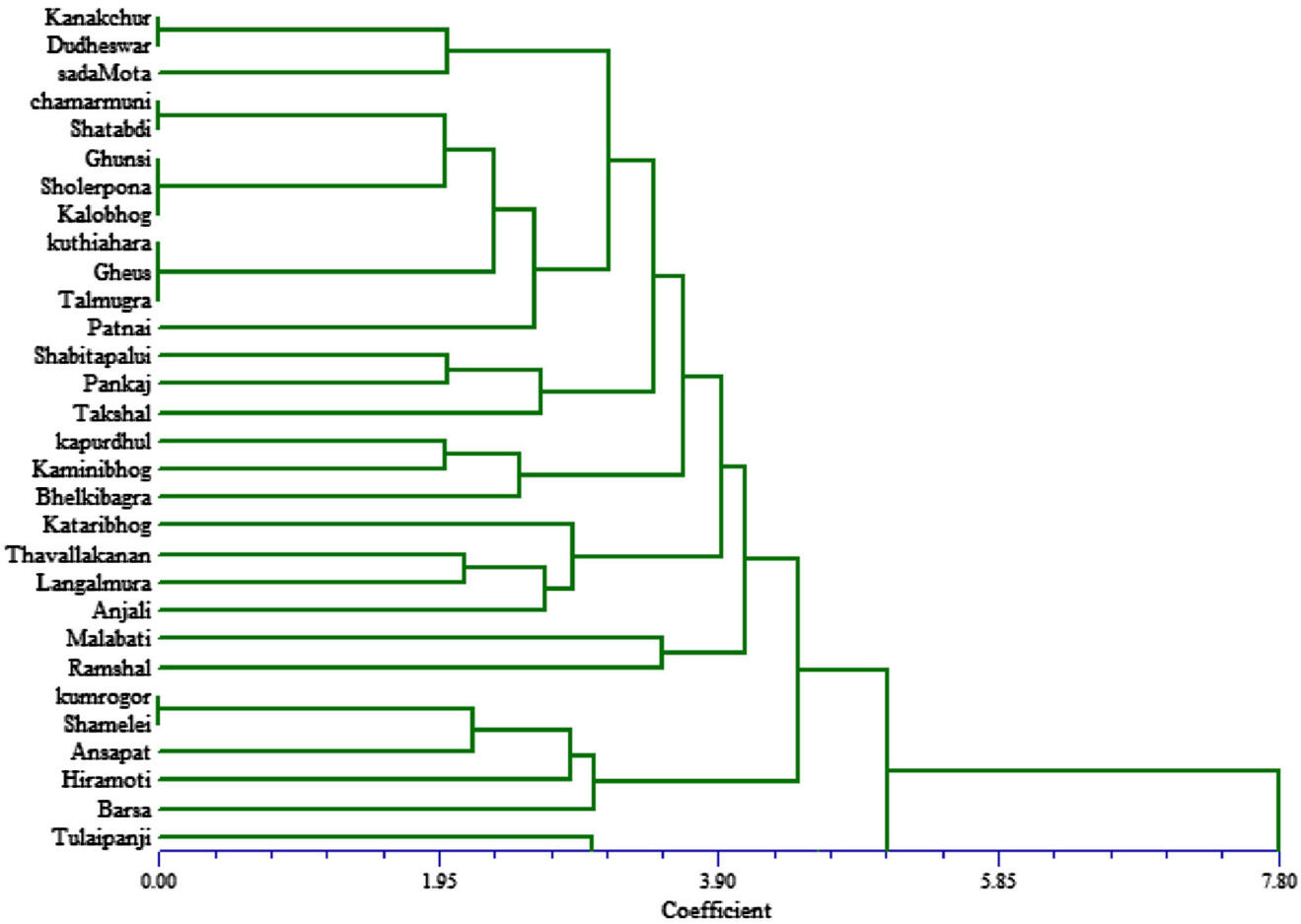

Fig. 5 Dendrogram based on SSR banding profile among 33 genotypes of rice under study

(2002), the advantages of using a multivariate analysis in the evaluation of salt tolerance are that it allows: (a) a simultaneous analysis of multiple parameters to increase the accuracy of the genotype ranking; (b) the ranking of genotypes even when plants are evaluated at different salt levels and salt tolerance varies with salinity levels, especially when the salt tolerance indices are averaged across salt levels; and (c) a more convenient and accurate estimation of salt tolerance among genotypes by simply adding the numbers in cluster group ranking at different salt levels. Because there is variation of salt tolerance among the seedling parameters for rice, the sensitive parameters, which can be single or multiple parameters, must be identified at different growth stages before using the cluster analysis.

Screening with SSR marker linked with salt tolerance

Eleven SSR markers tightly linked with salt tolerant QTLs present on chromosome number 1 and 6 were used for screening 33 genotypes of rice. One SSR marker viz. RM8007 did not give any amplification. The rest of the 10 SSR markers amplified polymorphic bands using 33 genotypes (Table 7) (Fig. 4, with RM 8053 as an instance). A total of 53 alleles were detected with these 10 primers. The allelic frequency of the major allele (as mentioned in Table 7) ranged from 0.21 (RM 7075 and RM 336) to 0.58 (RM 253).The lowest amplicon size belongs to RM 436 (81 bp) and the highest amplicon size belonged to RM 493 (300 bp). The number of microsatellite alleles of used markers ranged between 3 (RM 436) and 8 (RM 8094). Polymorphic information content (PIC) value varied from 0.55 to 0.84, the highest value belonged to RM 336, while RM 436 showed the lowest PIC value. The Marker Index (MI) was lowest in RM 436 (0.77) and it was highest in RM 8094 (1.92). The SSR markers RM 8094 was found to be the most superior for this analysis based on PIC coupled with MI value followed by RM 336 and RM 8046. Higher PIC with higher MI value indicates that all these primers were capable of distinguishing among genotypes and highly informative. This result was in conformity with the findings of Mohammadi-Nejad et al. (2008) and Aliyu et al. (2011).

On the basis of Euclidian distance matrix, 33 rice genotypes under study were grouped into 7 clusters (Fig. 5). The cluster composition is described here under Cluster 1: *Tulaipanji*, *Barsha*, *Hiramoti*, *Ansapat*, *Shyamali* and *Kumrogor*; Cluster

2: *Malabati* and *Ramshal*; Cluster 3: *Anjali*, *Langalmura*, *Thavalla Kanan* and *Kataribhog*; Cluster 4: *Bhelki Bagra*, *Kamini bhog* and *Kapur dhul*; Cluster 5: *Takshal*, *Pankaj*, *Shabita palui* and *Patnai*; Cluster 6: *Ghunsi*, *Sholerpona*, *Gheus*, *Talmugur*, *Kuthiahar* and *Kalohog*; Cluster 7: *Kanakchur*, *Dudheswar*, *Chamarmuni*, *Sadamota* and *Shatabdi*. The genotypes *Ghunsi*, *Sholerpona*, *Gheus* and *Kuthiahara* are always grouped with *Talmugur* (tolerant check) in the same cluster formed in the morphological and molecular clustering. This may help to conclude that these four genotypes may be considered as true salt tolerant genotypes.

The clustering pattern is different among these methods. Among these three methods of diversity analysis, molecular diversity provides the maximum genetic differences among the test genotypes followed by morphological. Several reports suggested that molecular diversity provides remarkably higher estimates of genetic diversity than morphological or physiological methods (Messmer et al. 1993; Karp et al. 1996; Beyene et al. 2005). Genotypes also swapped from one cluster to another cluster among different methods and this pattern is somewhat irregular. Differences in clustering pattern and swapping of genotypes among different clusters in different methods of diversity analysis have been reported in a number of studies (Suh et al. 1997, Han-yong et al. 2004, Thanh et al. 1999; Taran et al. 2005, Weiguo et al. 2007). These differences are not an indicator of the failure or limitation or weakness of the methods (Roldán-Ruiz et al. 2001). These results may be due to the diversity at the molecular level, which may not reflect in the diversity at the morphological or physiological level, as described by Karhu et al. (1996). To get similar diversity pattern among genotypes based on molecular and morphological diversity, the number of markers utilized in molecular analysis should be increased to several thousands and the morphological or physiological traits would contain all possible parameters. Another possible reason for this variation in clustering might be the environmental influence and genotype environment interaction. Morphological and physiological characters are the ultimate expression of molecular constitution of a variety where a number of biochemical processes are involved. So, different types of clustering in different methods are not unusual (Han-yong et al. 2004). Some studies have also warned of the dangers of assuming that marker-QTL linkage will remain in different genetic backgrounds or in different testing environments, especially for complex traits (Rohlf 1993). Even when a single gene controls a particular trait, there is no guarantee that DNA markers identified in one population will be useful in different populations, especially when the population originates from distinctly related germplasm (Rohlf 1993).

Conclusion

Highly significant differences among salinity concentrations and genotypes in seedling parameters were observed in this study. The interaction between salinity and genotypes was also statistically significant for seedling parameters measured at 12 days, after exposure of seedling in saline nutrient solution, indicating there are differential responses of genotypes to salinity from low to high levels. Genotype responses at different salinity levels were statistically different. Relative salt tolerance in terms of these three morphological parameters varied among genotypes. Shoot length and plant biomass were identified as better descriptors of tolerance index in comparison to root length. Out of 11 SSR markers, RM 8094, RM336 and RM 8046 with higher PIC coupled with higher Marker Index value are capable of distinguishing salt tolerance genotypes and are highly informative. They may be better descriptors to screen the salt tolerant genotypes. Interestingly, Hierarchical clustering using Euclidean Distance as distance measure for clustering suggested that the following genotypes were found promising genotypes for further breeding program in case of rice: *Talmugur*, *Gheus*, *Kumrogor*, *Langal mura*, *Shabita palui*, *Sadamota*, *Sholerpona*, *Ansapat*, *Ghunsi*, *Kataribhog*, *Kuthiahara*, *Malabati*, *Ramshal*. Combining morphological findings with that of the molecular assessment, the genotypes viz. *Gheus*, *Ghunsi*, *Kuthiahara* and *Sholer pona* may be considered as true salt tolerant genotypes which may contribute in a greater way in the development of salt tolerant genotypes in rice. The findings of our study will be helpful for both plant breeders and farmers of saline belts in general. For different saline belts having low to high salinity level, different genotypes may be recommended keeping their yield potential and response to salinity in mind. Twelve genotypes have been identified as resource base population, which could to be utilized suitably for further improvement programme for salt tolerance in rice. Plant breeders may give emphasis on shoot length and biomass during selection of salt tolerant genotypes of rice at seedling stage.

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